THE METABOLISM OF IRON-SULFUR CLUSTERS DURING HYDROGEN PEROXIDE STRESS IN *ESCHERICHIA COLI*

BY

SOOJIN JANG

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Doctoral Committee:

Professor James A. Imlay, Chair
Professor John E. Cronan, Jr.
Associate Professor Andrei Kuzminov
Professor James M. Slauch

ABSTRACT

An *Escherichia coli* strain that cannot scavenge hydrogen peroxide has been used to identify the cell processes that are most sensitive to this oxidant. Low micromolar concentrations of H_2O_2 completely blocked the biosynthesis of leucine. The defect was tracked to the inactivation of isopropylmalate isomerase. This enzyme belongs to a family of [4Fe-4S] dehydratases that are notoriously sensitive to univalent oxidation, and experiments confirmed that other members were also inactivated. In vitro and in vivo analyses showed that H_2O_2 directly oxidizes their solvent-exposed clusters in a Fenton-like reaction. The oxidized cluster then degrades to a catalytically inactive [3Fe-4S] form. In vitro experiments indicate that H_2O_2 accepts two consecutive electrons during the oxidation event; as a consequence, hydroxyl radicals are not released, the polypeptide is undamaged, and the enzyme is competent for reactivation by repair processes. Strikingly, in scavenger-deficient mutants in which H_2O_2 generated as an adventitious by-product of metabolism (< 1 μM) was sufficient to damage these [4Fe-4S] enzymes. This result demonstrates that aerobic organisms must synthesize H_2O_2 scavengers to avoid poisoning their own pathways.

However, the basal H_2O_2 scavengers are likely insufficient to protect organisms from exogenous H_2O_2 which results in several injuries including the oxidation of dehydratase [4Fe-4S] clusters. To protect itself, *E. coli* activates the OxyR regulon, including genes that encode the Suf iron-sulfur-cluster assembly system. *E. coli* normally relies on the Isc system for cluster assembly; however, when *E. coli* scavenger mutants were exposed to low-grade H_2O_2 stress, Suf was needed to maintain dehydratase activities. Experiments showed that Suf repaired damaged clusters in isopropylmalate

isomerase but not in fumarase. The reason was that damaged [3Fe-4S] clusters in IPMI were degraded in vivo, possibly to an apoprotein form, and thus required de novo assembly system for reactivation. In contrast, the [3Fe-4S] clusters in fumarase were stable and could be repaired by a simpler reduction/metallation process. Surprisingly, submicromolar H_2O_2 poisoned the Isc system, thereby creating a requirement for Suf both to repair IPMI and to activate Fe-S enzymes in general. The IscS and IscA components are H_2O_2-resistant, suggesting that oxidants disrupt Isc by oxidizing nascent Fe-S clusters as they are assembled on or transferred from the IscU scaffold. Consistent with these results, organisms that are chronically exposed to oxidants depend upon Suf rather than Isc for cluster assembly.

To my family

ACKNOWLEDGEMENTS

There are many people that I want to thank for helping me to finish this work. I would like to specially thank my advisor Dr. Jim Imlay. This work would have not been possible without him. He thought me how to be a better person as well as a better scientist. He will be always my role model.

I would like to thank my committee Dr. John E. Cronan, Dr. Andrei Kuzminov, and Dr. James M. Slauch for giving me truly helpful advice through my graduate school.

I would like to thank Dr. Mark Nilges for helping me to do EPR studies, Dr. Eric Massé for giving me a great opportunity to learn and contribute his work.

I would like to thank the people of the Imlay lab: Adil Anjem, Mianzhi Gu, Kari Imlay, Dr. Sergei Korshunov, Aedan Liu, Dr. Lee Macomber, Julia Martin, Dr. Surabhi Mishra, Dr. Sunny Park, Jason Sobota, Dr. Shery Varghese, Ann Wu and Fangfang Xu. They gave me unlimited love and support. I was truly blessed to be a part of this wonderful lab.

Finally, I would like to thank my family and friends for believing in me. I would have not come to U.S. and gotten a Ph. D. degree without their support and love. They always motivated me to be a better person and be a better scientist.

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CHAPTER 1: INTRODUCTION

1.1 WHAT ARE [FE-S] CLUSTERS?

About 3 billion years ago, when the first life emerged on Earth, oxygen was not present (predicted to beless than 0.001 % of the atmosphere) [12]. The anaerobic primitive earth provided a reduced environment in which iron and sulfur species were readily available. These chemically active iron and sulfur were useful for early organisms to conduct biological processes. Furthermore, iron and sulfur together formed a complex, termed an iron-sulfur cluster, in ancient proteins to catalyze cellular reactions. Iron-sulfur clusters still play important roles in modern organisms. Around 1960, when photosynthetic organisms and nitrogen-fixing bacteria were first intensively studied, researchers recognized the significance of iron-sulfur clusters [6, 93, 110]. During the last five decades, many studies have enlightened us about iron-sulfur clusters. However, we have not completely unveiled the 3 billion-year-old secrets of this ancient cofactor.

1.1.1 Structures

The basic structure of iron-sulfur ([Fe-S]) clusters is iron atom(s) that is(are) liganded to cysteinate sulfur atoms and inorganic sulfides. Based on the number of iron atoms in a cluster, it can be as simple as an $Fe(Cys)_4$ center, which is one iron atom with four cysteine residues, or can be more complicated structures such as an [8Fe-7S] cluster.

Other metals such as Mo and Ni are incorporated into [Fe-S] clusters in some cases [16, 125]. However, the most abundant cellular iron-sulfur clusters are [2Fe-2S] and [4Fe-4S] clusters.

1.1.2 Functions

As the diversity of their structures implies, [Fe-S] clusters are involved in many cellular processes in several different ways: electron transfer, Lewis acid-assisted enzyme catalysis, radical generation, sensors of environmental conditions, and stabilization of protein structures.

1.1.2.1 Electron transfer

The unique property of [Fe-S] clusters is its broad range of reduction potentials (-0.6 V ~ + 0.45 V) [14]. Different structures of [Fe-S] clusters have different reduction potentials. In addition, different residues in the neighborhood of clusters provide another layer of variation on reduction potentials. Small residue changes can tune reduction potentials of [Fe-S] clusters to an optimum range for various electron channels. For example, NADH dehydrogenase I (Ndh 1) in the *Escherichia coli* respiratory chain has nine [Fe-S] clusters. This enzyme transfers electrons from cytosolic NADH to ubiquinones in membranes. Figure 1.2 shows how [Fe-S] clusters are arranged in Ndh 1 [105]. Each cluster has a different reduction potential and is separated from neighboring clusters by 10 – 15 Å [97]. Once the enzyme gets an electron from NADH, the electron travels from one cluster to next, until it is ultimately transferred to ubiquinone.

[Fe-S] clusters can be found in many redox enzymes such as succinate dehydrogenase, fumarate reductase, sulfite reductase, and ferredoxin, to name a few.

Most electron carriers such as NAD, flavins, quinones, and hemes, have high reduction potentials. In contrast, the capability of [Fe-S] to have very low potentials makes them indispensible for low-potential coupling reactions carried out by enzymes such as hydrogenases, nitrogenases, and ferredoxins [66].

1.1.2.2 Dehydratases

[Fe-S] clusters in redox enzymes are buried inside polypeptides. On the other hand, [4Fe-4S] clusters in dehydratases have an iron atom that is exposed to solvent, while other three iron atoms are liganded to the polypeptide. The exposed iron atom serves as a substrate binding site and also as a catalytic site [66]. When substrate binds to the exposed iron atom in a [4Fe-4S] cluster, the iron center shifts from a tetrahedral to an octahedral structure as shown in Fig 1.3. Because of the geometric flexibility of an iron atom, this shift occurs without energy expense [47]. The cationic iron also catalyzes the dehydration reaction by retrieving an anionic hydroxyl group, acting as a Lewis acid, while a base deprotonates a methylene group. In this manner, [4Fe-4S] clusters in dehydratases are completely different from those in redox enzymes, which transfer electrons. Aconitase, fumarase, 6-phospogluconate dehydratase, isopropylmalate isomerase, and dihydroxyacid dehydratase are well known dehydratases that use solvent-exposed [4Fe-4S] clusters.

1.1.2.3 Radical enzymes

Pyruvate:formate lyase, and anaerobic ribonucleotide reductase are activated by activases. These activases are grouped in the SAM-radical superfamily along with biotin synthases, lipoate synthases, coproporphyrinogen III oxidase, and lysine 2,3-

aminomutase. The common feature of these enzymes is that they use adenosyl radicals to process high-energy demanding reactions such as metabolism of aliphathic substrates. Inert aliphathic metabolites can be activated by radicals [59, 79]. Although the principle seems simple, the actual generation of radicals is not. To produce adenosyl radicals, enzymes need to bind S-adenosylmethionine, which has a very low reduction potential, and give an electron to it. Nature found a [4Fe-4S] cluster to be ideally suited for this task. As in dehydratases, a [4Fe-4S] cluster in a SAM radical enzyme is exposed to solvent, which allows it to bind S-adenosylmethionine (Fig. 1.4). The subsequent coordination with a sulfur atom of the exposed iron atom is believed to depend upon an electron transfer from the cluster to SAM [18].

1.1.2.4 Sensors of environmental conditions

Sensing of changes in environments is an important strategy for cells to survive. [Fe-S] clusters are core players for sensing some stress conditions: iron limitation, dioxygen, and redox cycling drugs.

Iron is a physiologically essential metal. However, too much iron is toxic to cells. Therefore, cells monitor and control their intracellular iron concentration. To monitor iron concentrations, mammalian cells rely upon the iron regulatory protein, IRP-1 [37, 41, 103]. Surprisingly, an [Fe-S]- containing form of IRP-1 looks just like mitochondrial aconitase. During iron deficiency, cells cannot make [Fe-S] clusters. As a result, in that situation most IRP-1 proteins are in the apo-form. The apo IRP-1 then binds mRNAs that are related to iron homeostasis, thereby controlling their expression. For example, transferrin receptor is an iron import system. The mRNA of transferrin receptor has five binding sites for IRP-1 on its 3' region. Once IRP-1 binds

to the mRNA of transferrin receptor, it stabilizes the mRNA [61]. On the other hand, IRP-1 binds the 5' mRNA of ferritin, an iron-storage protein, and blocks its translation [61]. In summary, transferrin receptor is expressed more and the expression of ferritin is repressed during iron-starvation conditions.

Fumarate nitrate reductase regulator (FNR) is another example of a protein that uses a [Fe-S] cluster as a sensor [57, 71]. FNR stimulates the expression of genes that are involved in anaerobic metabolism. The mechanism is that anaerobically active FNR is a dimer that has two [4Fe-4S] clusters. These clusters are sensitive to oxygen molecules, so they are degraded aerobically [114]. As a consequence, the genes that are induced by FNR anaerobically, are repressed in aerobic environments.

A recent study suggests that SoxR senses redox-cycling drugs (M. Gu, unpublished data). A SoxR dimer has two [2Fe-2S] clusters that are redox-sensitive [32, 42]. Oxidized $[2Fe\text{-}2S]^{2+}$ clusters cause a conformational change of SoxR and induce the expression of SoxS, a transcriptional factor that induces other genes [32, 42, 126].

Interestingly, a single amino acid mutation in FNR decreases the rate of the cluster decay by at least 100-fold compared to wild type [58]. In addition, the similarity between IRP-1 and aconitase implies that development of an [Fe-S] cluster as a sensor might not be an evolutionarily huge challenge for cells [10, 38, 41, 103].

1.1.2.5 Stabilization of protein structures

As described before, [Fe-S] clusters have various architectures. This feature is important for some enzymes that need to be held in certain conformations. Endonuclease III is suggested to be one of these enzymes. It is believed that a [4Fe-4S] cluster in endonuclease III stabilizes the structure that is necessary to bind a specific

DNA site [116]. An amidotransferase of *Bacillus subtilis* needs a [4Fe-4S] cluster in order to be protected from proteases [35], and is another example that shows the stabilization of protein structures by [Fe-S] clusters. However, the stabilization of protein structures might not be the sole function of [Fe-S] clusters in these enzymes. More studies are needed to unveil their complete function.

Contemporary organisms from prokaryotes to eukaryotes still rely on [Fe-S] clusters to process many cellular reactions in the manners that are described above. The fact that 5 % of total proteins in *E. coli* are currently expected to contain [Fe-S] clusters highlights the significance of [Fe-S] clusters.

1.2 THE IRON-SULFUR CLUSTER ASSEMBLY SYSTEMS

During the first billion years in which the earth was still anaerobic, plenty of iron and sulfur were available, possibly allowing the spontaneous generation of [Fe-S] clusters [47]. The increased catalytic efficiency of the ancient proteins that obtained [Fe-S] clusters by chance would lead early organisms to use more [Fe-S] cluster enzymes. As evolution went on, the usage of [Fe-S] clusters expanded into important cellular processes. Therefore, it is not surprising that cells developed machinery devoted to the assembly of [Fe-S] clusters. Moreover, the anaerobic period ended as photosynthetic bacteria appeared and produced molecular oxygen, which can oxidize iron and sulfur. The eventual limitation of iron and sulfur made even more sense for cells to have [Fe-S] cluster assembly systems.

1.2.1 The mechanism of [Fe-S] cluster assembly

The function of the [Fe-S] cluster assembly system is not only building [Fe-S] clusters but also delivering them to recipient proteins. These tasks are too complicated to be performed by one protein. Therefore, a complex of functionally different proteins is responsible for [Fe-S] cluster assembly. The complex consists of an iron carrier, a desulfurase as a sulfur donor, a scaffold protein, proteins that are involved in cluster delivery, and accessory proteins [9]. The general mechanism is, first, a pyridoxal-phosphate (PLP)-dependent desulfurase retrieves sulfur from cysteine. Then, iron atoms and sulfur atoms from the desulfurase are transferred to a scaffold protein. These iron and sulfur atoms come together to form an [Fe-S] cluster, probably with the aid of electron donors. Second, a part of the assembly complex facilitates the delivery of the completed [Fe-S] cluster to a recipient protein (Fig. 1.5).

1.2.2 Types of assembly systems and their distribution in organisms

In the late 1980's, Dean and colleagues reported the Nif (nitrogen fixation) system that activates nitrogenase in *Azotobacter vinelandii* [31, 50]. A mature nitrogenase in *A. vinelandii* has a MoFe cofactor ($MoFe_7$-S_9) (5). Dean's group found that the mutation of the Nif system (*nifSU*) hampered maturation of nitrogenase [50, 55, 132]. The Nif system was the first indication of the existence of [Fe-S] cluster assembly systems. Soon, people realized that the mutation of the Nif system does not completely eliminate the nitrogenase activity, which implies that there is another way to activate nitrogenase. The Isc (iron sulfur cluster) system was subsequently discovered [133]. A phylogenetic study revealed that the Isc system is distributed in most proteobaceria and in the mitochondria of eukaryotes [118]. A third assembly system, the so-called Suf

(sulfur mobilization) system, was also found [115]. Surprisingly, phylogenetic analysis showed that the Suf system is present in a diverse set of organisms including most eubacteria, archaea, plants, and parasites [118] (Fig. 1.6). The reports of [Fe-S] cluster biogenesis from bacteria promoted the study of their eukaryotic counterparts. [Fe-S] cluster enzymes in eukaryotes are found in three compartments: the cytosol, mitochondria, and the nucleus [74]. Therefore, it has been a question if each compartment has its own [Fe-S] cluster assembly system. So far, the working model is that the mitochondrial Isc system is responsible for [Fe-S] cluster biogenesis in all compartments [9, 52, 78, 86, 112]. Recently, a second system was found, the CIA (cytosolic iron-sulfur cluster assembly) system [6, 7, 40, 104]. However, the function of this system requires the mitochondrial Isc system.

1.2.3 [Fe-S] cluster assembly systems in *Escherichia coli (E. coli)*

The fundamental cellular reactions and the related genes are generally similar in all organisms. Therefore, researchers often study simple organisms, which can be easily handled, as a model system to understand more complicated organisms. *E. coli* has been a favorite model organism, since advanced genetic tools and the knowledge about its physiology are available. The biogenesis of [Fe-S] clusters has been studied in *E. coli* in depth. *E. coli* has two major [Fe-S] cluster assembly systems: the Isc and Suf systems [8, 9, 29]. Besides these two systems, recent genetic analyses discovered additional individual proteins that are involved in cluster assembly [1, 3, 77, 84].

1.2.3.1 The Isc system

The Isc system in *E. coli* is the house-keeping assembly system during normal growth. It is encoded by a seven-gene operon: *iscRSUA-hscBA-fdx*. IscR is an autoregulator for the transcription of *iscRSUA*. Holo IscR contains a [2Fe-2S] cluster and decreases the expression of IscRSUA by acting as a repressor [107]. On the other hand, the depletion of [Fe-S] clusters renders IscR in the apo-form, causing the derepression of IscRSUA expression [96]. The second gene in the *isc* operon encodes a desulfurase, IscS. Mutation of *iscS* causes growth defects and reduced activity of [Fe-S] cluster enzymes, including aconitase, 6-phosphogluconate dehydratase, glutamate synthase, fumarase, and NADH dehydrogenase [25, 67, 106]. These results indicate that IscS is the major source of sulfur for [Fe-S] cluster assembly under normal conditions. Genetic analysis also revealed that IscS is a sulfur donor for thiolation process, such as tRNA modification [54, 68].

Crystal structure, electron paramagnetic resonance (EPR), and Mössbauer spectra analyses indicated that homodimer IscU contains [2Fe-2S] and/or [4Fe-4S] clusters [2, 17, 109]. In addition, in vitro biochemical studies showed that an [Fe-S] cluster can be transferred from holo IscU to apo ferredoxin, which suggests that IscU serves as a scaffold protein [91, 94, 129]. The function of IscA is debatable. In vitro data have suggested that IscA could harbor [2Fe-2S]/[4Fe-4S] clusters or an iron atom [23, 24, 87, 94, 131]. Therefore, it could be either a secondary scaffold protein or an iron carrier. In vivo studies are required to answer this question.

Long before their functions in [Fe-S] cluster biogenesis were recognized, HscA and HscB were identified as members of the Hsp70 and Hsp40 chaperone families [110, 124]. An *hscA* mutation decreases the activity of [Fe-S] cluster enzymes as low as an

iscS mutation does, indicating the essential role of HscA in [Fe-S] cluster assembly [25]. Currently, HscA/HscB are thought to facilitate the transfer of a [Fe-S] cluster from IscU to a recipient protein by hydrolyzing ATP [20, 43, 111]. The last member of the Isc system is ferredoxin (*fdx*), containing a [2Fe-2S] cluster. Like other *isc* mutants, an *fdx* mutant has growth defects and the reduced activity of [Fe-S] cluster enzymes [25]. The nature of the ferredoxin family leads to an idea that Fdx donates an electron at some point during [Fe-S] cluster biogenesis. However, the specific in vivo role of Fdx still remains to be unveiled.

1.2.3.2 The Suf system

Since the discovery of the Isc system, Tokumoto and his colleagues sought mutations that suppress deletion of the Isc system. Most of the suppressors had mutations that could potentially induce the *suf* operon (*sufABCDSE*) [115]. In subsequent experiments, they found that the overexpression of SufABCDSE complements most defects in the *isc* deletion mutation [115].

SufA, the first member of the Suf system, shares 40% amino acid sequence identity with IscA [94]. As the similarity suggests, SufA is able to assemble [2Fe-2S] or [4Fe-4S] clusters in vitro [93, 94]. Moreover, assembled [Fe-S] clusters can be transferred to apo recipients [94]. The role of SufA, however, is unclear because of a lack of in vivo data. The proteins encoded by the following three genes, *sufBCD*, are thought to make a complex ($SufBC_2D$) [92, 95, 102]. SufC is an atypical member of the ABC-ATPase superfamily [60]. Unlike other ABC transporters, the SufBCD complex localizes in the cytosol, which raises an intriguing question about the function of its ATPase activity [92, 95]. Since SufBCD is postulated to be a scaffold, it may work as

the ATP dependent IscU/HscAB complex does. The Suf system also contains a desulfurase, SufS. The poor desulfurase activity of SufS in early studies puzzled researchers [84, 85]. Subsequent studies revealed that another member of the Suf system, SufE, binds to SufS and increases the SufS desulfurase activity to a level comparable to IscS [76, 93, 95, 117]. Therefore, SufSE together functions as a true desulfurase. Moreover, the addition of the SufBCD complex increases SufSE activity even higher [70]. Unlike IscS, SufSE does not intervene in tRNA modification [56].

1.2.3.3 The regulation of the Isc and Suf system

The general function of the Isc and Suf systems seems redundant. A question would be why *E. coli* has the Suf system, since, unlike *isc* mutations, *suf* mutations did not significantly change the activity of [Fe-S] cluster enzymes [25]. Differences in regulation suggest that Isc and Suf may be expressed under different conditions. As described before, the Isc system is regulated by the pool of [Fe-S] clusters, which IscR senses [33, 107]. The global iron response regulator, Fur (ferric uptake regulator) indirectly regulates the Isc system [82, 96, 99]. When the intracellular iron concentration is low, the Fur regulon, including RyhB, is derepressed [80]. Then RyhB, a small RNA, targets mRNAs of iron-metabolism genes for degradation [81]. In the case of the Isc system, RyhB binds an untranslated region between *iscR* and *iscS* and triggers the degradation of *iscSUA* mRNA [22] (Fig. 1.7A). Similarly, expression of the Suf system is controlled by IscR and Fur. However, the mechanisms differ. Unlike the Isc system, apo-IscR actively binds to the promoter of the *suf* operon and induces its transcription [73, 130]. Instead of RyhB-mediated regulation, iron-metallated Fur directly represses Suf expression [73, 96]. On top of the regulation by IscR and Fur,

hydrogen peroxide stress that is sensed by OxyR induces the expression of Suf [72, 96, 134] (Fig. 1.7B). In summary, when the demand for [Fe-S] clusters increases, both the Isc and Suf systems are induced. However, during iron depletion, the Isc system is repressed, while the Suf system is induced. Moreover, OxyR induces the Suf system, but not the Isc system, during H_2O_2 stress. These different induction conditions suggest that the Suf system might be important during iron starvation and H_2O_2 stress. It has been reported that the Suf system is essential under iron-deficient conditions [96].

1.2.3.4 Other proteins that are involved in [Fe-S] cluster assembly

A third desulfurase, CsdA (cysteine sulfinate desulfurase), was discovered by a search of the *E. coli* genome [84]. Although the highest in vitro desulfurase activity of CsdA compares to those of IscS and SufS, no obvious phenotype was observed in a *csdA* mutant [64]. Recently, a possible role of CsdA in [Fe-S] cluster assembly, which is mediated by SufBCD, was postulated [119]. However, since the experiment was done with overexpressed CsdA from a plasmid, its involvement in [Fe-S] cluster biogenesis is still not clear.

Frataxin is a protein that is conserved from prokaryotes to eukaryotes [44]. In humans, a deficiency of frataxin causes Friedreich's ataxia, a neurodegenerative disorder [101]. Research on Yfh1, a yeast homologue, revealed that the deletion of *YFH1* causes decreased activity of [Fe-S] cluster enzymes as well as the accumulation of iron in mitochondria, which suggests that Yfh1 has a role in [Fe-S] cluster synthesis [5, 19, 26, 30, 90, 127]. The current working model is that Yfh1 is an iron carrier. *E. coli* also has a frataxin homologue, CyaY. Although CyaY shows iron-binding capacity in vitro and interaction with IscS in vivo, no phenotypes have been observed for *cyaY* mutant [1,

69]. ErpA and NfuA are so-called A-type scaffolds, as are IscA and SufA [3, 77]. The functions of ErpA and NfuA are believed to be similar to those of IscA and SufA. The hypothesis is that each A-type scaffold delivers [Fe-S] clusters to a specific subset of recipient proteins. More in vivo studies are needed to test this hypothesis.

1.3 PROBLEMS OF [FE-S] CLUSTERS DURING OXIDATIVE STRESS

1.3.1 Oxidative stress: H_2O_2 and O_2^-

Oxidative stress is caused by reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals (•OH). These reactive oxygen species are toxic to all living organisms. There is growing support for the idea that reactive oxygen species are implicated in human diseases including cancer as well as aging. Therefore, oxidative stress has received enormous attention. The major questions are the source of ROS and their toxic mechanisms.

1.3.1.1 Sources of H_2O_2 and O_2^-

The existence of scavengers for H_2O_2 and O_2^- in most living organisms indicates that most creatures must face these reactive oxygen species (ROS). Indeed, aerobically growing cells are inevitably exposed to ROS, which are generated endogenously. Endogenous production of ROS occurs when oxygen adventitiously steals an electron from redox enzymes such as flavoenzymes [83](Fig. 1.8). This phenomenon happens proportionally to the collision frequency of O_2 with a flavin [48]. Therefore, the rate of H_2O_2 and O_2^- generation is proportional to the intracellular oxygen concentration.

This information implies that higher organisms, whose cells are mostly microaerobic, might produce less endogenous H_2O_2 and O_2^-. In *E. coli*, the estimated rate of O_2^- generation is 5 μM/s. The rate of H_2O_2 production was experimentally measured using a H_2O_2 scavenger mutant. It is approximately 15 μM/s [108]. Although H_2O_2 and O_2^- are continuously generated during aerobic growth, scavenging enzymes successfully maintain the intracellular concentration of these species below the toxic concentration [46].

However, cells must also deal with poisonous ROS that are from other organisms. Some organisms take advantage of H_2O_2 and O_2^- toxicity and use them as a biological weapon. For example, a macrophage in a mammalian immune system, produces H_2O_2 and O_2^- to kill invaders during phagocytosis [62, 65]. Lactic-acid bacteria also produce over 1 mM H_2O_2 to protect their habitat from competitors [49]. In other cases, some plants and bacteria excrete redox-cycling antibiotics, which can produce ROS when they are imported by competitors [39, 128]. Therefore, aerobically growing cells have to confront the ROS challenge because there is no way to avoid it.

1.3.1.2 Targets of H_2O_2 and O_2^-

What are the targets of H_2O_2 and O_2^-? For decades, researchers have tried to answer this question. Despite the enormous effort, it has not been an easy task. The reason was that cells have evolved to defend themselves from H_2O_2 and O_2^- attacks. Therefore, researchers often had to add non-physiological doses (a few millimolar) of H_2O_2 to create toxicity.

The construction of H_2O_2 or O_2^- scavenger mutants solved this problem. *E. coli* peroxidase/catalase mutants accumulates approximately 1 μM H_2O_2 during aerobic

growth and show growth defects [51, 98]. This result indicates that, surprisingly, 1 μM H_2O_2 is enough to poison cells. O_2^- toxicity was observed in *E. coli* cytosolic superoxide dismutase (SOD) mutants. The SOD mutations also caused aerobic phenotypes [63].

These scavenger deficient mutants showed that H_2O_2 and O_2^- share a few common targets. First, DNA damage occurs during both H_2O_2 and O_2^- stress. The damage mechanism is that H_2O_2 reacts with DNA-bound ferrous iron and produces •OH via the Fenton reaction (Reaction 1). Then, the highly reactive •OH attacks DNA [39].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\bullet \qquad \text{(Reaction 1)}$$

The rate constant of the Fenton reaction is 5000-20,000 $M^{-1}s^{-1}$ [98](115). This rate constant is fast enough for less than 1 μM H_2O_2 to create a lethal amount of DNA damage in cells. O_2^- indirectly increases DNA damage by elevating the concentration of intracellular iron [27].

Another common target is the $[4Fe\text{-}4S]^{2+}$ cluster in dehydratases [28, 98]. As described before, the [4Fe-4S] cluster in a dehydratase is exposed to solvent. Therefore, it may be naturally vulnerable to oxidation, especially by small oxidants such as H_2O_2 and O_2^-. This hypothesis was confirmed by monitoring the activity of dehydratases in SOD mutants. Over 50% of aconitase B activity decreased in SOD mutants compared to wild-type cells [123]. The rate of inactivation by O_2^- was measured as 10^6 $M^{-1}s^{-1}$ using purified enzymes [28]. The proposed inactivation mechanism is that O_2^- attacks the solvent-exposed iron in a $[4Fe\text{-}4S]^{2+}$ cluster and then converts the $[4Fe\text{-}4S]^{2+}$ cluster to a $[3Fe\text{-}4S]^{+}$ cluster releasing Fe^{2+} [28]. Unlike the O_2^- case, the result of damage to

[4Fe-4S] clusters by H_2O_2 was somewhat arguable. The reasons are first, in vivo and in vitro experiments used physiologically irrelevant concentrations of H_2O_2, 4 mM and 300-600 μM respectively [25, 28]. Second, in vitro data might not illuminate in vivo situations since purified [Fe-S] cluster enzymes are extremely sensitive to oxidation even by molecular oxygen [13]. Therefore, in vivo experiments using physiological concentrations of H_2O_2 are required to confirm that H_2O_2 damages [4Fe-4S] clusters in dehydratases.

Both SOD and peroxidase/catalase mutants also are not able to synthesize aromatic amino acids [11](122, J Sobota, unpublished data). The mechanism is not yet clear. Recent data suggest that H_2O_2 inactivates ribulose phosphate 3-epimerase (Rpe) of the pentose phosphate pathway (J. Sobota, unpublished data). It seems that only iron-metallated Rpe is inactivated by H_2O_2. Another phenomenon, which appears during H_2O_2 stress, is the derepression of the Fur regulon [122]. It could be because of either the direct oxidation of Fur-bound ferrous iron or the limitation of intracellular ferrous iron, which is caused by H_2O_2. Unlike H_2O_2 stress, O_2^- stress causes a problem with sulfur metabolism [15]. The mechanism of this problem is under investigation.

As described above, most known phenotypes arise from the Fenton-like reaction of Fe. Therefore, it is important to investigate iron related reactions as potential targets of ROS.

1.3.1.3 Defense mechanisms against H_2O_2

When a problem occurs, logically, the first response would be to relieve the problem by eliminating the cause and then mending any damage. Nature follows the same logic to deal with H_2O_2 toxicity.

The primary cellular defense against H_2O_2 is removing H_2O_2 by scavengers. Since Oscar Loew reported the existence of catalases in 1900, other H_2O_2 scavengers have been reported in a broad range of organisms [48, 75, 113]. Catalases (*katG* and *katE*), which dismute H_2O_2 (Reaction 2), and peroxidase (*ahpCF*), which reduces H_2O_2 (Reaction 3) are the major H_2O_2 scavengers in *E. coli*.

$$H_2O_2 + \quad H_2O_2 \rightarrow 2\ H_2O + O_2 \qquad \text{(Reaction 2)}$$

$$NADH + H_2O_2 \rightarrow NAD^+ + 2\ H_2O \qquad \text{(Reaction 3)}$$

Both *katG* and *ahpCF* are induced during H_2O_2 stress by OxyR [134]. OxyR is the global regulator which responds to H_2O_2. DNA microarray data revealed that OxyR regulates at least 20 genes, including *katG* and *ahpCF* [134] (Table 1).

One of the members of the OxyR regulon is *dps* (DNA-binding protein from starved cells). Dps is a nonspecific DNA-binding protein which has a similar structure to ferritin. As its structural similarity to ferritin indicates, Dps sequesters iron atoms (~ 4500) per molecule and prevents the Fenton reaction. As a result, it diminishes DNA damage during H_2O_2 stress [98].

MntH, a manganese transporter, is also induced by OxyR. In vitro data that showed direct scavenging of H_2O_2 by manganese led researchers to misunderstand the defense mechanism of manganese. Recent work demonstrates that manganese does not scavenge H_2O_2 in vivo [4]. The proposed defense mechanism is that manganese might displace iron from metalloenzymes. Therefore, H_2O_2-sensitive enzymes that were metallated with iron become resistant. This hypothesis is supported by recent data that

show resistance of manganese-metallated Rpe to H_2O_2, unlike iron-metallated Rpe (J. Sobota, unpublished data).

Many genes in the OxyR regulon, not surprisingly, have been characterized as defense system against H_2O_2. Uncharacterized members are also thought to be potentially related to the detoxification of H_2O_2. The fact that the Suf system is regulated by OxyR implies the involvement of the Suf system in the cellular defense against H_2O_2 [134]. Some interesting questions are why cells need the Suf system and what the role of the Suf system is during H_2O_2 stress.

Cells also have defense proteins that are independent of OxyR, such as hydroperoxidase II (*katE*), exonuclease III (*xthA*), DNA polymerase I (*polA*), RecA, and methionine sulfoxide reductase (*msrA*). All of these proteins play roles in either antioxidant or repair systems involved in combating H_2O_2 stress [21, 45, 48, 88, 89].

1.3.1.4 Defense mechanisms against O_2^-

The primary defense mechanism against O_2^- is performed by superoxide dismutases (SOD), which catalyze Reaction 4.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Reaction 4)}$$

E. coli has two cytoplasmic SODs and one periplasmic SOD: MnSOD, FeSOD, and CuZnSOD, respectively. Aerobically growing cells are estimated to produce O_2^- at the rate of 5 μM/s. SODs are believed to maintain the steady-state O_2^- concentration at 0.1 nM [46].

In 1990, Demple's and Weiss' groups independently found a two-component system that responds to redox stress: SoxRS [36, 120]. SoxR senses redox stress and activates SoxS. SoxS is a transcription factor that activates about twenty genes [100] (Table 2). Since *sodA* (MnSOD) is in the SoxS regulon, it has been thought that SoxRS responds to O_2^- stress.

Dehydratases' [4Fe-4S] clusters are well known targets of O_2^-. Therefore, it would make sense that the SoxS regulon includes genes for defense against to damage to those clusters. Indeed, dehydratase isozymes (*fumC* and *acnA*) that are resistant to O_2^- are in the SoxS regulon [100, 121, 123].

Since $[3Fe\text{-}4S]^+$ clusters are proposed to be the products of inactivation of $[4Fe\text{-}4S]^{2+}$ clusters by either H_2O_2 or O_2^-, one could imagine that there is a repair mechanism. Presumably, a reduction of $[3Fe\text{-}4S]^+$ to $[3Fe\text{-}4S]^0$ followed by an addition of ferrous iron would repair the cluster. In vivo data showed that when H_2O_2 and O_2^- stresses ceased, inactivated dehydratases regained their activity even without new protein synthesis. Neither the Isc system nor the Suf system was responsible for the repair. A new study has implicated YggX, a member of the SoxS regulon, in the repair process [34]. Another protein that might play a role in cluster repair is YtfE [53]. YtfE is regulated by neither OxyR nor SoxRS. The repair mechanisms of YggX and YtfE remain unclear.

Although, for a long time, people have believed SoxRS to be a O_2^- sensor, recent data suggests that SoxRS primarily responds to redox-cycling drugs instead of O_2^- (M Gu, unpublished data).

1.4 SCOPE OF THIS THESIS

1.4.1 Solvent exposed [4Fe-4S] clusters are primary targets of H_2O_2

It has been shown that [4Fe-4S] clusters in dehydratases are sensitive to O_2^-. Based on this result, damage to [4Fe-4S] clusters by H_2O_2 was tested in vivo and in vitro. Although the results supported the hypothesis, it was not clear if the results were physiologically relevant because the experiments were performed using high doses of H_2O_2. Therefore, we decided to test the hypothesis using physiological concentrations of H_2O_2 (a few micromolar). This work clarified most arguable results from previous reports and reaffirmed that H_2O_2 damages [4Fe-4S] clusters in dehydratases at the rate constant of 4×10^4 $M^{-1}s^{-1}$. Furthermore, we have proposed a new model for the damage mechanism.

1.4.2 H_2O_2 and O_2^- inhibit the function of the Isc system

The patterns of regulation of the Isc and Suf systems suggest that the Suf system might be a defense system against H_2O_2 stress. This assumption led us to hypothesize that the Isc system is inactivated by H_2O_2. As a result, the Suf system may be essential during H_2O_2 stress. We found that H_2O_2 inactivates the Isc system and that the inactivation of the Isc system is reversible. Moreover, our data suggest that O_2^- also inactivates the Isc system.

1.4.3 The Suf system is essential to compensate for the loss of the Isc system during H_2O_2 and O_2^- stresses

Subsequently, we postulated that the Suf system is important when the Isc system is inactivated. We found that the Suf system compensates for the loss of the Isc system by performing de novo synthesis of [Fe-S] clusters and repairing damaged enzymes.

1.5 TABLES

Table 1.1 Members of the OxyR regulon

Role	Enzyme
H_2O_2 scavenging	ahpCF katG
Heme synthesis	Ferrochelatase
[Fe-S] cluster assembly	SufABCDSE
Iron scavenging	Dps
Iron-import control	Fur
Divalent cation import	MntH
Disulfide reduction	Thioredoxin C Glutaredoxin A Glutathione reductase DsbG (periplasmic reductase)
Unknown function	Several

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Table 1.2 Selected genes induced by the SoxRS system

Oxidant-resistant dehydratase isozymes	*fumC* (fumarase C) *acnA* (aconitase A)
Suspected cluster repair	*yggX* (Fe/S cluster repair protein ?) *zwf* (glucose-6-phosphate dehydrogenase) *fpr* (NADH:flavodoxin/ferredoxin oxidoreductase) *fldA* (flavodoxin A) *fldB* (flavodoxin B)
Drug efflux and/or resistance	*acrAB* (drug efflux pump) *tolC* (OMP component of drug efflux pump) *micF* (OmpF antisense sRNA) *marAB* (multiple antibiotic resistance operon) *nfnB* (nitroreductase) *rimK* (modification of ribosomal protein S6)
Other	*nfo* (endonuclease IV) *fur* (iron-uptake regulatory protein) *sodA* (manganese-containing superoxide dismutase) *ribA* (cGMP hydrolase)

Imlay JA (2008) *Annu Rev Biochem* **77**:755-76

1.6 FIGURES

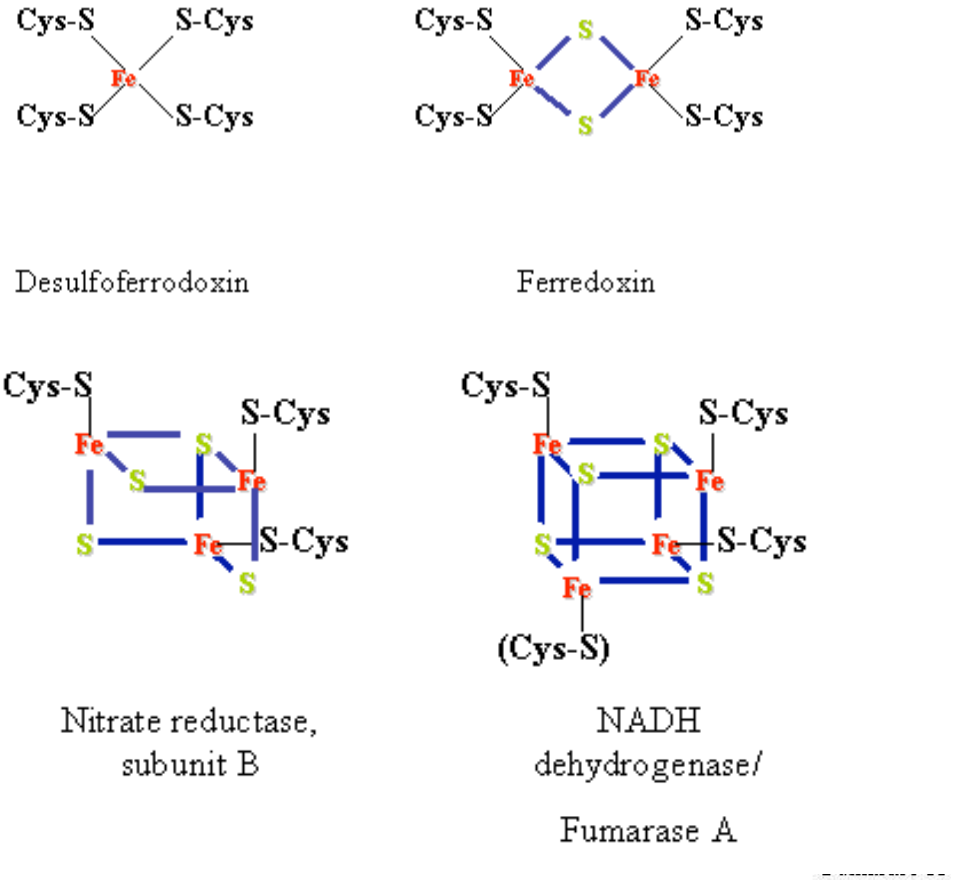

Figure 1.1 Various types of iron-sulfur clusters and examples.

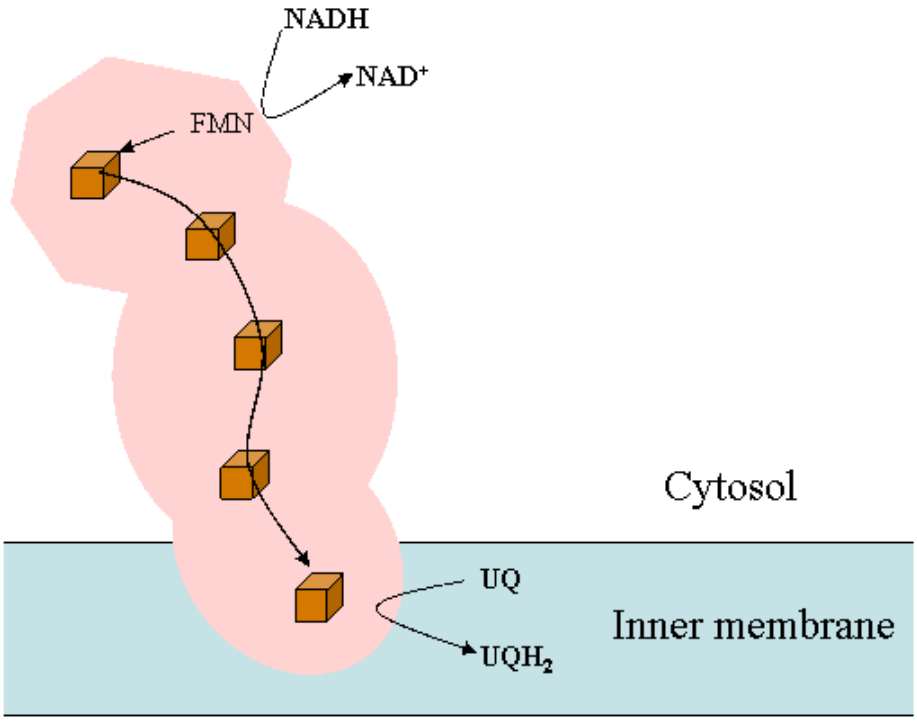

Figure 1.2 NADH dehydrogenase I.

Sazanov, L. A. and Hinchliffe, P.(2006) *Science* 311:1430-1436.

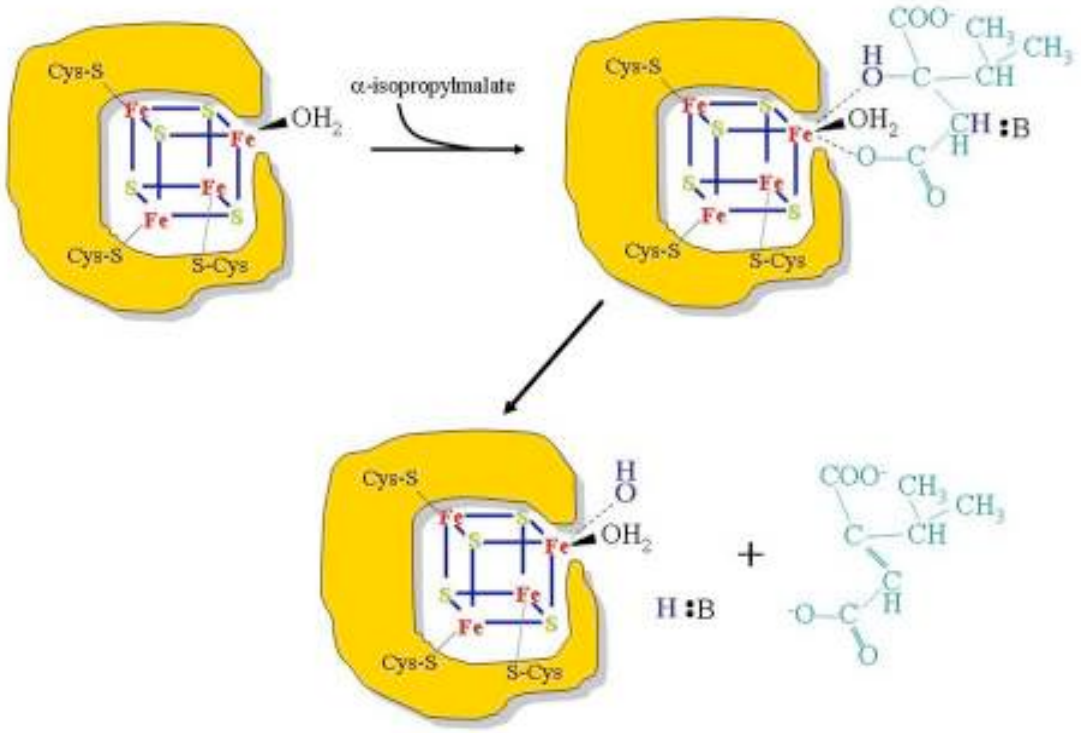

Figure 1.3 Isopropylmalate isomerase (IPMI).

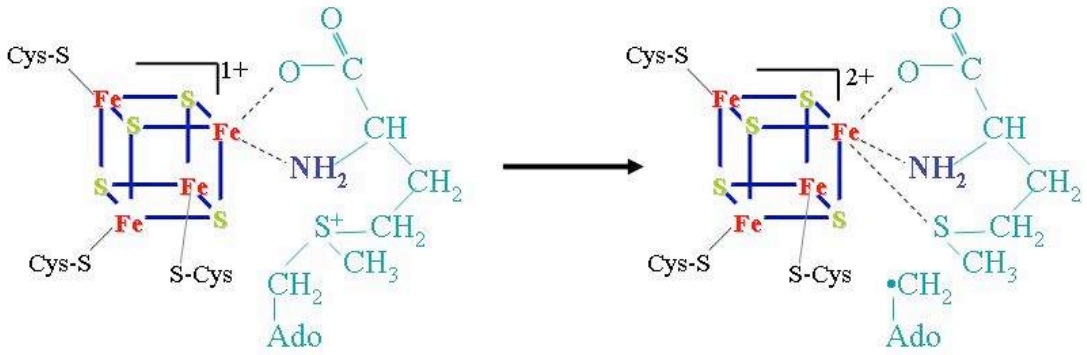

Figure 1.4 Proposed S-adenosylmethionine binding and scission in lysine 2,3-aminomutase (Chen *et al*., 2003).

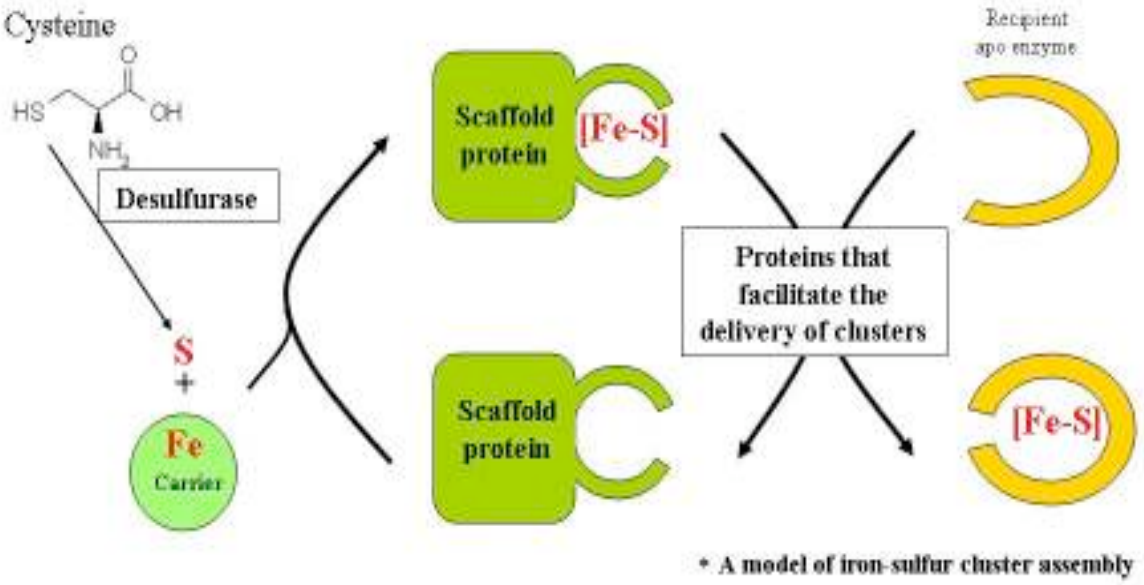

Figure 1.5 Iron-sulfur cluster assembly system.

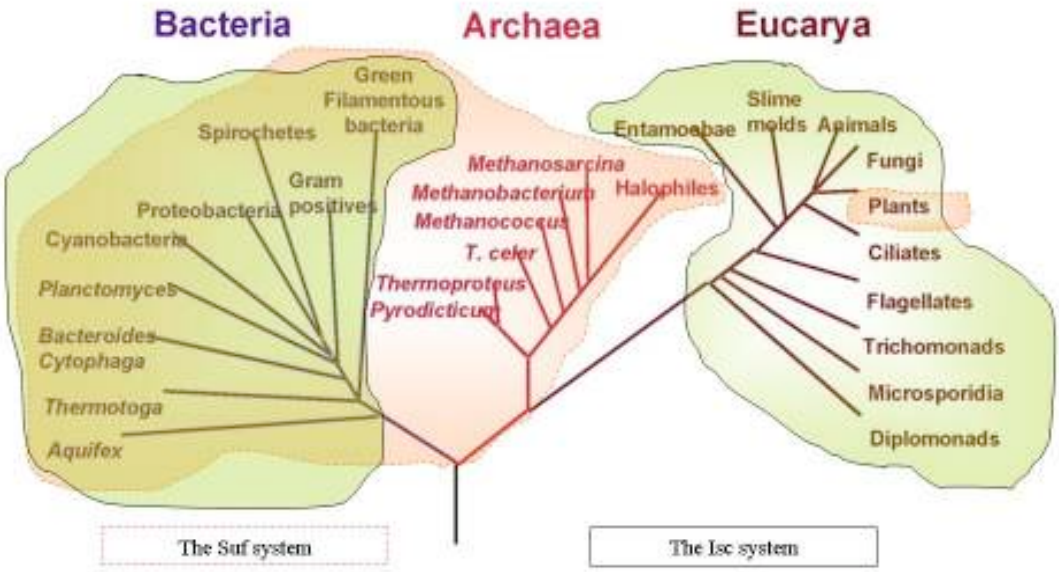

Figure 1.6 The phylogenetic tree of the Isc and Suf systems.

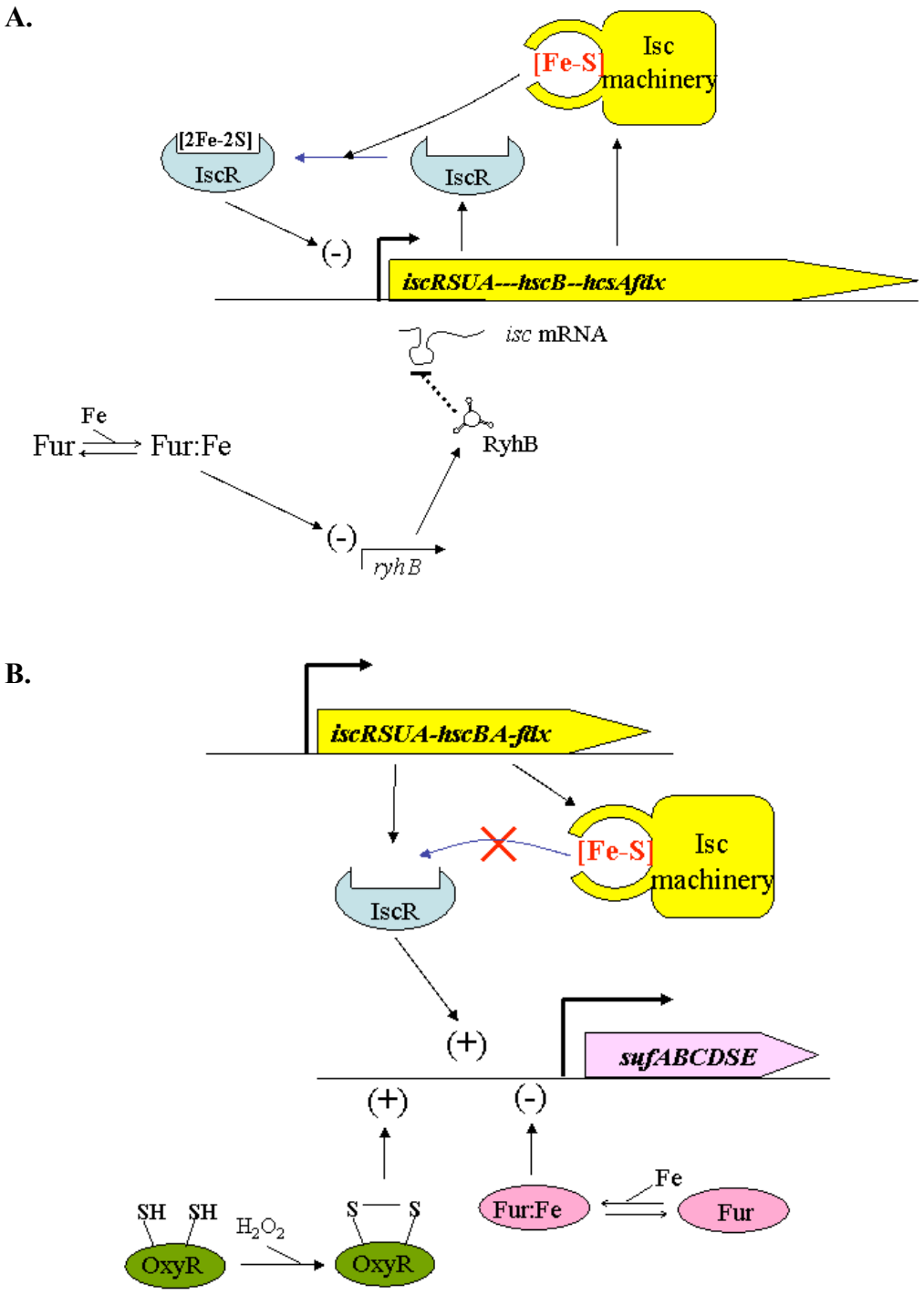

Figure 1.7 (A) The regulation of the *isc* operon by IscR and RyhB. (B) The regulation of the *suf* operon by OxyR, IscR, and Fur.

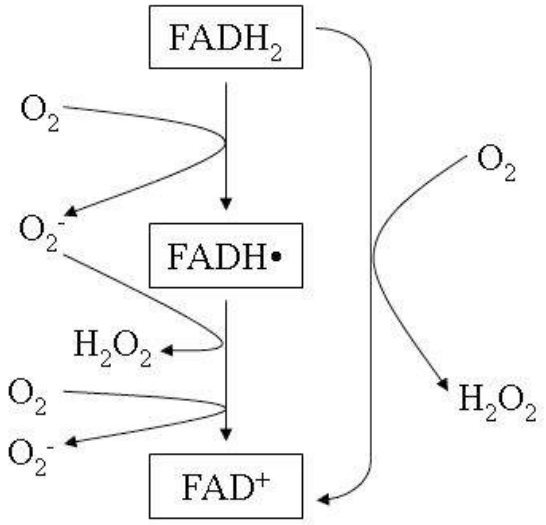

Figure 1.8 The production of reactive oxygen species on a flavoenzyme.

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CHAPTER 2: MICROMOLAR INTRACELLULAR HYDROGEN PEROXIDE DISRUPTS METABOLISM BY DAMAGING IRON-SULFUR ENZYMES

2.1 INTRODUCTION

Virtually all organisms express peroxidases and catalases to protect themselves from hydrogen peroxide. H_2O_2 is continuously formed by the autoxidation of redox enzymes (reviewed in [16]), and scavenging enzymes may have originally evolved to protect cells against these internal sources of H_2O_2. The peroxidases and catalases are sufficiently abundant and active that they probably drive the steady-state level of intracellular H_2O_2 into the low nanomolar range [38]. Nevertheless, it is widely suspected that even this dose of H_2O_2 may comprise a chronic, low-level stress that gradually debilitates cells and, in higher organisms, drives the deterioration of tissue function as part of the aging process.

Exogenous H_2O_2 rapidly diffuses across cell membranes [37] and can impose a much more acute stress on cells; accordingly, it is often used as a biological weapon. For example, H_2O_2 is formed by phagocytes and may accumulate to 10^{-4} M inside phagosomes that have engulfed invading bacteria. Lactic acid bacteria suppress the growth of competitors by releasing H_2O_2 as a primary metabolic product, achieving millimolar concentrations in lab cultures. And redox-cycling antibiotics, which are produced as microbicides both by plants and bacteria, suffuse target organisms with a continuous stream of H_2O_2.

If we wish to understand the severity and nature of the stress that H_2O_2 imposes upon cells, we must identify the biomolecules with which it primarily reacts. This

problem has not been easy to solve. In vitro studies have shown that H_2O_2 can oxidize methionine [13] and cysteine [43] residues, but the rates with which it does so suggest that these types of damage will be scant at physiological doses of H_2O_2, unless the surrounding polypeptide context somehow strongly activates the residues. Reactions between H_2O_2 and loosely bound iron generate hydroxyl radicals and are suspected of being involved in protein carbonylation, lipid peroxidation, and DNA oxidation [14]:

$$Fe^{2+} + H_2O_2 \rightarrow HO^{\cdot} + OH^{-} + Fe^{3+} \quad \text{(Eq. 1)}$$

Early measurements indicated that this reaction (the Fenton reaction) is relatively slow as well [41], prompting some workers to question its significance in real-world scenarios (discussed in [14]); however, subsequent work revealed that anionic ligands activate ferrous iron to the point that it reacts quickly with micromolar H_2O_2 [33, 36].

An alternative approach to pinpointing the important targets of H_2O_2 is to expose cells to increasing doses in a way that identifies the first cell processes to fail. *E. coli* and other organisms have calibrated their defensive systems to detect submicromolar levels of H_2O_2 [3, 38], so we anticipate that these low concentrations are sufficient to threaten the most sensitive biomolecules. Unfortunately, if scavenging enzymes are active, it is difficult to impose such a low dose of H_2O_2 over an extended period of time, since the enzymes will degrade the H_2O_2 and end the stress. Therefore, these experiments are most easily conducted with scavenger-deficient mutants. We have constructed *E. coli* strains that lack peroxidase and catalase activities [37]. These mutants grow at wild-type rates in anaerobic environments, but when they are exposed to oxygen they grow at reduced rates in complex medium, and they fail to grow at all in a

minimal medium. The former defect is due, at least in part, to Fenton-mediated DNA damage [33]. The second defect stems from problems with biosynthetic pathways. In this study we identify the mechanism by which micromolar H_2O_2 blocks leucine biosynthesis, and we find that this class of injury affects multiple pathways in the cell.

2.2 MATERIALS AND METHODS

2.2.1 Strains and culture conditions

Strains and plasmids used in this study are listed in Table 2.1. Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratory Products, Inc.), and aerobic cultures were grown with vigorous shaking in a water bath at 37°C. Standard minimal medium contained minimal A salts [30], 0.2% glucose, 1 mM $MgCl_2$, 5 mg/liter thiamine, and 0.5 mM each of histidine, phenylalanine, tyrosine, and tryptophan. Histidine was always added to the media because the parent strain, MG1655, is a histidine auxotroph anaerobically; to minimize the difference between anaerobic and aerobic cultures, histidine was also added to aerobic cultures. Where indicated, lactose (0.2%), gluconate (0.2%), or malate (40 mM) were substituted for glucose. Where indicated, other amino acids, α-ketoisovalerate (TCI America), or α-ketoisocaproate (Sigma) were present at 0.5 mM.

Mutations were introduced into new strains by P1 transduction [30]. To create a null mutation of *fumB* using the Red recombinase method [6], the forward primer 5'-TAACAAATACAGAGTTACAGGCTGGAAGCTGTAGGCTGGAGCTGC-3' and the reverse primer 5'-AGCATGCTGCCAGGCGCTGGGCCGAAGAGGATATGAATATCC TCC-3' were used. Mutations were confirmed by PCR analysis and enzyme assays.

2.2.2 Aerobic cell growth

To ensure that cells were growing exponentially before they were exposed to oxygen, anaerobic overnight cultures of Hpx$^-$ cells were diluted to an OD_{600} of 0.005 in fresh anaerobic minimal glucose medium. Cells were then grown anaerobically to an OD_{600} of approximately 0.1 prior to dilution into aerobic medium.

2.2.3 Enzyme assays

Cell extracts were prepared by suspending and sonicating cells in anaerobic buffers inside an anaerobic chamber. Isopropylmalate isomerase (IPMI) activity was measured by monitoring the decrease in the absorbance (235 nm) of citraconate (Sigma) [21], an analogue of isopropylmalate; reactions contained 100 mM Tris-Cl (pH 7.6) and 0.4 mM citraconate. Fumarase activity was determined from the appearance of fumarate (250 nm) in a reaction containing 50 mM sodium phosphate (pH 7.4) and 50 mM malate (Sigma) [28]. To assay 6-phosphogluconate dehydratase, lysates were prepared from cultures grown in a minimal medium containing 0.2% gluconate. Turnover of 6-phosphogluconate dehydratase produces pyruvate; its formation (in 50 mM Tris-Cl, pH 7.65) was determined in a second reaction catalyzed by lactate dehydrogenase (Sigma) [12]. To assay NADH dehydrogenase I (Ndh1), inverted membrane vesicles were isolated from extracts after sonication in 50 mM MES, pH 6.0 [29]. NdhI oxidizes deaminoNADH (340 nm) in 50 mM potassium phosphate buffer (pH 7.8), whereas NADH dehydrogenase II does not. Aconitase was assayed by the conversion of isocitrate to aconitate [40].

2.2.4 Plasmid construction

The *leuCD* ORF was PCR-amplified from *E. coli* MG1655 by using the forward primer 5'-ATATCGAATTCTTAACGATATCATTGCCCGCTATATAGCAG-3' and the reverse primer 5'-CTGGATCTAGATTAATTCATAAACGCAGGTTG-3'. To construct the pLEUCD2 plasmid, the PCR products were digested with XbaI and EcoRI and cloned into pWKS30 [42] vector behind the *lac* promoter. The plasmid was confirmed by restriction analysis and by the isopropylmalate isomerase assay. Hpx^- cells containing pLEUCD2 or pWKS30 were cultured in lactose minimal medium with histidine, aromatic amino acids, and 50 μg/ml ampicillin. This plasmid was used in complementation experiments; the *leuCD* genes were induced three-fold above wild-type levels by 1 mM IPTG, which was added to both anaerobic precultures and final aerobic cultures.

For EPR studies and purification purposes, IPMI and fumarase A were strongly overproduced by expression from a *tac* promoter. The *leuCD* genes were excised from pLEUCD2 with XbaI and EcoRI and cloned into pCKR101 vector to construct pLEUCD3. The *fumA* ORF was PCR-amplified from *E. coli* MG1655 by using the forward primer 5'-ATATCGAATTCTTAACATAACCAAACCAGGCAGTAAGTG-3' and the reverse primer 5'-CATGGATCTAGATTATTTCACACAGCGGGTGCATTG-3'. PCR products were digested with XbaI and EcoRI and cloned into the pCRK101 vector to construct pFUMA. Cells containing the plasmids were cultured in glucose minimal medium with histidine, aromatic amino acids, and 50 μg/ml of ampicillin. These plasmids overproduced IPMI and fumarase A more than 60-fold above wild-type levels.

2.2.5 Inactivation of enzymes

Hpx$^-$ cells were grown anaerobically to 0.2 (OD_{600}). H_2O_2 was added to the cultures when they were aerated. At time points, aliquots were removed, catalase was added to 200 U/ml, and cells were returned to the anaerobic chamber for lysis and assay.

In vivo inactivation of IPMI by endogenous H_2O_2 was initiated by aerating heretofore anaerobic cultures without any addition of exogenous H_2O_2. Under these conditions the cells steadily generate H_2O_2. The H_2O_2 equilibrates so quickly across membranes that in Hpx$^-$ cultures the intracellular H_2O_2 concentration is essentially equivalent to the extracellular concentration [37]. The extracellular H_2O_2 was measured directly by the amplex red/horseradish peroxidase method [38]. At the same time, extracts were prepared and assayed in the anaerobic chamber.

The inactivation of enzymes in vitro was accomplished by the addition of H_2O_2 to lysates or to purified enzyme in anaerobic buffer. The H_2O_2 was subsequently removed by catalase prior to anaerobic assay. In some cases, damaged iron-sulfur clusters were chemically rebuilt by incubation with 50 μM $Fe(NH_4)_2(SO_4)_2$ (Sigma) and 2.5 mM dithiothreitol [40] (Sigma) at room temperature.

2.2.6 EPR analysis

In vivo EPR samples were prepared with Hpx$^-$ cells that overproduce IPMI or fumarase A. To overexpress the structural genes, 1 mM IPTG was added when cells reached an OD_{600} of 0.2. After another 2 hours of incubation, the cells were harvested by centrifugation, and the cell pellets were resuspended in 1/500th of original culture volume in 10 % glycerol. The resuspended cells were incubated with H_2O_2 for 1 min at 37°C. The cell suspension (250 μl) then was transferred into an EPR tube and frozen in

dry ice. EPR spectra of $[3Fe\text{-}4S]^{+}$ clusters [7] were obtained with the following settings: microwave power, 1 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 8 Gauss at 100 KHz; time constant, 0.032.

2.2.7 Purification of enzymes

Four-liter cultures of the Hpx^{-} mutant that overproduces fumarase A were grown anaerobically to an OD_{600} of 0.2 at 37°C. Expression of *fumA* was induced by incubation with 1 mM IPTG for 2 hours at 37°C. The cultures were harvested by centrifugation, the cell pellets were resuspended in 15 ml of anaerobic 50 mM Tris-Cl/10 mM Mg^{2+} (pH 8) and disrupted by sonication, and cell debris was removed by centrifugation. The purification procedure was derived by the protocol described by Flint [8]. All steps of the purification were conducted in an anaerobic chamber at room temperature, and all buffers were anaerobic. Protamine sulfate (1 %) was added to the supernatant to remove nucleic acids. The supernatant was then loaded onto a DEAE-Sepharose column (16 mm by 140 mm) and eluted by a 0–1 M gradient of KCl in 50 mM Tris-Cl/10 mM Mg^{2+}. The fractions containing the highest activity were pooled. Ammonium sulfate (1.5 M) was added, precipitate was removed by centrifugation, and the supernatant was loaded onto a phenyl sepharose column (16 mm by 110 mm). The fractions were eluted by a 1.5 – 0 M gradient of ammonium sulfate in 50 mM Tris-Cl/10 mM Mg^{2+}. The fractions containing the highest activity were pooled and concentrated (Amicon). The enzyme was loaded onto a Superdex column (16 mm by 50 mm). The fractions were eluted with 500 mM KCl in 50 mM Tris-Cl/10 mM Mg^{2+}. The three fractions containing the highest activity were frozen in dry ice containing ethanol. Purified fumarase A was more than 90% pure based on SDS-PAGE. The enzyme was

quantified by dye-binding assay using bovine serum albumin as a standard and the conversion factor determined by Flint [8].

Aconitase A was overproduced and purified as described [40].

2.2.8 Detection of iron released upon cluster oxidation

EPR spectroscopy was used to quantitatively correlate the creation of $[3Fe\text{-}4S]^+$ clusters with the release of iron when FumA was damaged in vitro. Desferrioxamine (1 mM, Sigma) was added to purified fumarase A (18 μM) immediately prior to the addition of H_2O_2. The sample was then transferred into an EPR tube and frozen. The $[3Fe\text{-}4S]^+$ signal was detected as described above, while EPR spectra of free iron were obtained with the following settings: microwave power, 10 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032. Iron concentrations were quantified using standard solutions of $FeCl_3$ (Sigma) in 50 mM Tris-Cl containing 10 mM Mg^{2+} and 1 mM desferrioxamine. Desferrioxamine binds both ferric and ferrous iron and triggers the oxidation of the latter species. Thus the EPR method did not distinguish whether the released iron was in the ferric or ferrous form.

Ferene is commonly used to detect ferrous iron that is released by metalloenzymes, but we found that ferene itself directly inactivated fumarase A. Dipyridyl did not. Dipyridyl binds ferrous iron in a complex that exhibits an absorbance maximum at 522 nm [27]; we determined that this complex cannot be oxidized by H_2O_2. To detect Fe^{2+} that is released during cluster decomposition, 1 mM dipyridyl (Sigma) was added to 18 μM of fumarase A prior to H_2O_2 addition. Control experiments confirmed that this concentration of dipyridyl captured ferrous iron before the H_2O_2 could oxidize it: when $Fe(NH_4)_2(SO_4)_2$ was added to the H_2O_2-containing

reaction mixture, we were able to quantitatively recover it as a dipyridyl chelate, and we could accurately detect as little as 2 μM.

2.2.9 Protein mass spectroscopy

Fumarase A (3 μM) was treated with 5 μM H_2O_2 for 2 min at room temperature. The reaction was terminated by the addition of 0.1 U/μl catalase. Assays showed that >98 % of the enzyme had been inactivated. For mass spectroscopic analysis, 2 μg of sample was desalted using the Genotech (St. Louis) Perfect Focus 2-D sample Clean up kit. The desalted sample was suspended in 25 mM ammonium bicarbonate containing 12.5 μg/ml trypsin and was incubated for 12 hours at 37 ^{o} C. The sample was then dried, suspended in 5% acetonitrile/0.1% formic acid, and injected into a quadrupole time-of-flight mass spectrometer (Waters Q-ToF) via an HPLC interface. Peptide masses were detected using a data-dependent method and were subjected to MS/MS for de novo sequencing and characterization of post-translational modifications. Analysis was done using Waters Protein Lynx Global Server 2.1, MASCOT (Matrix Sciences), and PEAKS (Bioinformatics Solutions, Inc.).

2.3 RESULTS

2.3.1 H_2O_2 damages isopropylmalate isomerase (IPMI) and causes a leucine auxotropy in hydroperoxidase mutants (Hpx^-)

Hydroperoxidase mutants that cannot scavenge H_2O_2 (*katG katE ahp*, here denoted Hpx^-) grow well in anaerobic glucose minimal medium but stop growing when they are aerated. The cells resume growth when aromatic amino acids are supplied,

indicating that endogenously formed H_2O_2 poisons some step in the aromatic biosynthetic pathway. The mechanism is unknown and is the subject of a separate investigation. However, aromatic supplements do not fully restore these mutants to a wild-type growth rate, and the addition of small amounts of exogenous H_2O_2 exacerbates the residual defect. Eight micromolar H_2O_2 competely blocked growth in this medium (Fig. 2.1A). Supplementation with casamino acids restored growth, implying that stasis was a result of a second amino-acid biosynthetic defect. Experiments using different amino acids determined that leucine was the critical one.

Hpx^- growth in the presence of H_2O_2 was improved by supplementation with exogenous α-ketoisocaproate, the final intermediate in the leucine pathway, but not by α - ketoisovalerate, the first one. There are five reactions between the two intermediates, catalyzed by four enzymes (Fig. 2.2). We anticipated that H_2O_2 might inhibit or inactivate one of them. Indeed, the growth defect was partially relieved by a plasmid that overproduces isopropylmalate isomerase three-fold (Fig. 2.1B), suggesting that this is the rate-limiting enzyme in the pathway during H_2O_2 stress.

Enzyme assays confirmed that isopropylmalate isomerase (IPMI) lost activity rapidly when 8 μM H_2O_2 was added to cultures (Fig. 2.3A). Furthermore, significant enzyme inactivation occurred upon aeration even without the addition of exogenous H_2O_2 (Fig. 2.3B), indicating that < 0.4 μM intracellular H_2O_2 was sufficient to poison a substantial fraction of IPMI. Thus this enzyme is exquisitely sensitive, and scavenging enzymes are needed to protect it from endogenously formed H_2O_2.

2.3.2 The nature of IPMI damage

IPMI activity was lost when H_2O_2 was added to anaerobically prepared Hpx^- extracts (Fig. 2.4A), indicating that inactivation occurs by direct action of H_2O_2 upon the enzyme. Catalase protected completely (data not shown). IPMI is a dehydratase, and its protein sequence suggests that it belongs to family of enzymes that employ [4Fe-4S] clusters as active-site catalysts [15]. The solvent-exposed clusters of these enzymes both coordinate substrate and act as Lewis acids in abstracting the hydroxide anion from the bound substrate [23]. These enzymes are typified by aconitase, and they are notoriously sensitive to inactivation by univalent oxidants such as superoxide and ferricyanide [9, 11, 12, 22, 25]. These agents abstract a single electron from the cluster, converting it to a $[4Fe\text{-}4S]^{3+}$ form. The cluster is unstable in that valence and releases the substrate-binding iron atom as Fe^{2+}, so that the residual cluster is left in a $[3Fe\text{-}4S]^{+}$ form that lacks the key iron atom and is catalytically inactive.

Consistent with this model, the addition of citraconate, a pseudosubstrate of IPMI, protected the enzyme from H_2O_2 in vitro, suggesting that H_2O_2 must directly contact the cluster in order to inactivate the enzyme. Damaged clusters can often be reassembled chemically by treatment with dithiothreitol and ferrous iron; when damaged IPMI was subjected to this protocol, 60% of the activity was regained within three minutes (Fig. 2.4B).

IPMI was overexpressed inside Hpx^- cells, and cells were then exposed to H_2O_2. A strong $[3Fe\text{-}4S]^{+}$ signal appeared (Fig. 2.4C) which was absent from non-overproducing controls. This result confirmed both that IPMI has a cluster and that it is destroyed by H_2O_2. In fact, *ca.* 85% of the IPMI activity was recovered when the extracts from H_2O_2-exposed cells were treated with dithiothreitol and ferrous iron.

2.3.3 Other [4Fe-4S] dehydratases are similarly sensitive to H_2O_2

Other cluster-containing dehydratases—6-phosphogluconate dehydratase and fumarases A and B—also lost activity when they were exposed to low concentrations of H_2O_2 in vitro (Fig. 2.5A, B, and C). In each case full activity could be restored by subsequent treatment with dithiothreitol and ferrous iron (Fig. 2.6). Further, a strong $[3Fe\text{-}4S]^+$ signal was detected when H_2O_2 was added to Hpx^- cells that overproduced fumarase A (Fig. 2.7). Thus it appears that H_2O_2 efficiently damages the clusters of all members of this enzyme family.

Glucose medium, which we employed for our initial growth studies, does not demand that *E. coli* process substrate through its TCA cycle in order to generate ATP; therefore, growth would not have been affected by inactivation of aconitase or fumarase. However, the Hpx^- strain exhibited a severe growth defect when it was cultured in malate medium (Fig. 2.8). This result indicates that the submicromolar H_2O_2 that is generated by endogenous processes is sufficient to debilitate this pathway.

Iron-sulfur clusters are also used by respiratory enzymes to transfer electrons between active sites. The NADH dehydrogenase I complex is characteristic of these enzymes, as it contains at least 9 Fe-S clusters [32]. However, even 5 mM H_2O_2 was unable to diminish its activity. Because these clusters are buried within polypeptide, the implication is that H_2O_2 can only oxidize those clusters which it can contact directly.

2.3.4 The mechanism of cluster inactivation

IPMI is a two-subunit enzyme that dissociates during purification. Therefore, fumarase A and aconitase A were chosen for purification and further examination. The

isolated enzymes were acutely sensitive to H_2O_2, exhibiting inactivation rate constants of 4×10^3 M^{-1} s^{-1} and 3×10^2 M^{-1} s^{-1}, respectively, at 0^o C. The fumarase inactivation constant was 0.5-1 x 10^5 M^{-1} s^{-1} at 25^o C, and inactivation at 37^o C was too fast for us to measure.

Strikingly, the fumarase rate constants were orders of magnitude higher than the apparent constant that we calculated in vivo using Hpx^- mutants. We suspected that substrates protect the enzyme inside the cell. Indeed, both malate and fumarate fully protected fumarase when they were added in saturating concentrations (Fig. 2.9). The doses needed for half-maximal protection (0.4 mM for malate and 0.3 mM for fumarate, measured at 0^o C) were in reasonable agreement with the Km's of the enzyme for those substrates (0.7 and 0.6 mM respectively, at 37^o C [10]). Thus it is likely that the inactivation of the enzyme in vivo is tempered by the consequent accumulation of substrate. These data also support the suspicion that H_2O_2 must intimately contact the cluster in order to inactivate it.

The standard model of univalent cluster oxidation posits that a $[3Fe\text{-}4S]^+$ form is initially generated with release of one ferrous ion [9]. However, if the oxidant is H_2O_2, then a hydroxyl radical should also be formed.

$$[4Fe\text{-}4S]^{2+} + H_2O_2 \rightarrow [4Fe\text{-}4S]^{3+} + OH^- + HO^{\cdot} \qquad \text{(Eq. 2)}$$

$$[4Fe\text{-}4S]^{3+} \rightarrow [3Fe\text{-}4S]^{+} + Fe^{2+} \qquad \text{(Eq. 3)}$$

Hydroxyl radicals react with most organic biomolecules, including amino acids, at nearly diffusion-limited rates; therefore, if one were formed within the active site of a

dehydratase, the likely consequence would be the direct oxidation of the protein. However, we were able to quantitatively reactivate H_2O_2-treated fumarase (data not shown), which seemed inconsistent with the oxidation of active-site residues. SDS-PAGE analysis of the protein showed that the polypeptide chain was not cleaved. Mass spectroscopy failed to detect any oxidation of histidyl, lysyl, prolyl, or methionyl residues (data not shown).

An alternative model is that a ferryl-like species is generated by the initial electron transfer from the cluster to liganded H_2O_2, and that the ferryl radical then abstracts a second electron from the cluster rather than release a hydroxyl radical:

$$[4Fe\text{-}4S]^{2+} + H_2O_2 \rightarrow [4Fe\text{-}4S/O]^{2+} + H_2O \qquad \text{(Eq. 4)}$$

$$[4Fe\text{-}4S/O]^{2+} + H^+ \rightarrow [3Fe\text{-}4S]^{+} + Fe^{3+} + OH^- \qquad \text{(Eq. 5)}$$

To test this possibility, we used desferrioxamine and dipyridyl to quantify the release of total iron and ferrous iron, respectively. Ferrous iron is generated by the first mechanistic scheme (eq. 3) but not by the second (eq. 6). While we detected stoichiometric release of one iron atom per cluster (Fig. 2.10), none of this iron was in the ferrous form (< 0.1 atom/cluster) (data not shown). From this result, and from the absence of polypeptide oxidation, we infer that a free hydroxyl radical is not generated.

Previous experiments, which were conducted with millimolar doses of oxidant, indicated that with time the cluster disintegration might continue past the $[3Fe\text{-}4S]^+$ state [7]. However, the ease with which we were able to chemically reactivate damaged dehydratases was inconsistent with the formation of apoprotein, which is only slowly

reactivated. In fact, EPR analysis showed that the $[3Fe\text{-}4S]^+$ fumarase cluster was unaffected by a ten-minute exposure to 100 μM H_2O_2 (Fig. 2.10A). We conclude that over this period physiological doses of H_2O_2 do not directly degrade clusters beyond the easily repaired $[3Fe\text{-}4S]^+$ state.

2.4 DISCUSSION

The experiments reported here reveal that sub-micromolar concentrations of H_2O_2 are sufficient to destroy enzymic iron-sulfur clusters. Multiple catabolic and biosynthetic pathways are thereby disrupted. Earlier work with the same strain demonstrated that the endogenous H_2O_2 also reacts with unincorporated iron to produce high levels of DNA damage [33]. The two phenotypes are of a piece in that both result from Fenton-type reactions.

The sensitivity of Fe-S clusters to univalent oxidants has been appreciated for some time; in fact, much of the toxicity of superoxide is due to inactivation of the same enzymes that were identified in this study. Damage by millimolar doses of H_2O_2 has been reported [4, 40]. However, while the chemistry is not unexpected, the surprise is that these reactions occur so rapidly. The rate constant of the Fenton reaction was measured to be 76 M^{-1} s^{-1} at pH 3, and this number has been cited widely [41]. It carried with it the implication that reactions between iron and H_2O_2 are too slow to occur at an important rate in most biological scenarios, where H_2O_2 concentrations are micromolar or lower. However, more-recent measurements have shown that at physiological pH the rate constant for hexaqueous iron is actually orders of magnitude higher, presumably because at neutral pH hydroxide anion coordinates iron and lowers its

reduction potential. Extrapolating from measurements made at lower temperatures (8), we estimate that the rate may be 20,000-30,000 M^{-1} s^{-1} at 37° C. The same enhancement evidently pertains to the substrate-binding iron atom within a dehydratase iron-sulfur cluster.

The biological consequence of this high reactivity is that far less H_2O_2 is needed to create toxicity than had been suspected. The data directly show that *E. coli* must synthesize scavenging enzymes to avoid being poisoned by the high-nanomolar H_2O_2 that it makes through the adventitious oxidation of its own redox enzymes. Basal levels of the NADH peroxidase solve that problem. However, this basic vulnerability is still exploited by phagocytes and plants, both of which suppress the growth of invading bacteria by generating far higher levels of H_2O_2, either directly or through the synthesis of redox-active antibiotics [17, 20, 35, 44].

These deliberate oxidative assaults, as well as the H_2O_2 that accumulates in the environment through chemical oxidation processes, are a true threat to bacteria. Because H_2O_2 influx across membranes can outstrip the action of scavenging enzymes, extracellular concentrations of 5 μM H_2O_2 are sufficient to raise the intracellular H_2O_2 in wild-type (scavenger-proficient) cells to about 1 μM [38]. Therefore, as little as 5 μM of environmental H_2O_2 is sufficient to enzyme and pathway defects that were reported in this study.

Interestingly, lactic acid bacteria employ a similar strategem to inhibit competing organisms, elaborating lactate and pyruvate oxidases which can drive H_2O_2 levels in their immediate environment up to millimolar levels [18, 34]. How do the lactic acid bacteria themselves tolerate the stress that they are creating? Lactic acid bacteria do not rely upon pathways that contain Fe-S dehydratases. Instead, they obtainfrom their

environment the amino acids that dehydratase-dependent biosynthetic pathways would normally produce, and they ferment sugars to lactic acid rather than using the TCA cycle.

To defend themselves against such H_2O_2-mediated assaults, microbes throughout the biotic world have evolved H_2O_2-inducible defenses, most of which are either homologues or analogues of the OxyR system of *E. coli*. The front-line defense of these systems is the strong induction of scavenging enzymes, but the repair of damaged clusters is also an important feature. Ongoing cluster repair may contribute to the disparity between the rates of enzyme damage that we measured in vitro and the apparent rates that we determined in vivo. In previous studies we had observed that cells quantitatively repair H_2O_2-damaged dehydratases [7], but we were perplexed as to why the hydroxyl radical, which presumably had been formed upon cluster oxidation, did not irreversibly damage active-site amino-acid residues. This puzzle is resolved by the observation that ferric (rather than ferrous) iron is released during cluster oxidation. Therefore a ferryl radical--or a non-diffusing hydroxyl radical--evidently pulls a second electron from the residual cluster rather than allowing an unexpended hydroxyl radical to be released into the active-site bulk solution. Interestingly, an analogous phenomenon was noted for PerR protein of *Bacillus subtilis*, in that a Fenton reaction between its prosthetic ferrous iron atom and H_2O_2 caused immediate oxidation of the iron ligands, rather than the more widely distributed oxidation of the polypeptide as might have been expected from a diffusible hydroxyl radical [24].

In the dehydratase case we do not stipulate whether the second electron transfer would generate an intermediate all-ferric $[4Fe\text{-}4S]^{4+}$ cluster or if it would create a disulfide bond concomitant with ferric iron release. However, in either case polypeptide oxidation would be avoided. The physiological significance is that the enzyme can be

restored to its native state by cellular processes that reduce and remetallate the $[3Fe\text{-}4S]^{+}$ cluster. The components that catalyze that repair process have not yet been identified. However, the OxyR system responds to H_2O_2 by inducing the Suf proteins [46], which comprise a backup system for cluster assembly.

A secondary consequence of cluster damage is that iron is released in an uncontrolled manner into the cytosol. Loose iron can bind to DNA and catalyze the formation of damaging hydroxyl radicals; therefore, this iron leak has the effect of accelerating the rate at which H_2O_2 produces DNA damage [19, 26, 39]. The OxyR system addresses this threat by inducing the synthesis of Dps, a ferritin-like storage protein that scavenges loose iron and sequesters it into an unreactive ferric hydroxide core [1, 2, 33, 45]. Thus it has become clear that several aspects of the inducible defense against H_2O_2 are well-matched to the threat that H_2O_2 poses.

2.5 TABLES

Table 2.1 Strains and plasmids

Strain or plasmid	Genotype or characteristics	Source or ref.
MG1655	Wild type *E. coli*	[31]
LC106	*Δ(katG17::Tn10)1 (ahpC-ahpF')del kan::'ahpF Δ(katE12::Tn10)1*	[37]
JH400	*Δ(dgs-fumCA-manA)D1 Δ(mel-fumB) zdg232::Tn10, metB1,spoT1, relA*	John Guest
OD570	*Δ fumC::Cm*	[7]
OD571	*Δ fumCA::Cm*	[7]
SJ19	*Δ fumC::Cm in LC106*	This study
SJ20	*ΔfumAC::Cm in LC106*	This study
SJ34	*ΔfumB::Cm in BW25113*	This study
SJ37	*ΔfumC and ΔfumB::Cm in LC106*	This study
SJ38	*ΔfumAC and ΔfumB::Cm in LC106*	This study
BW25113	*F- λ- lacI^q rrnB$_{T14}$ ΔlacZ$_{WJ16}$ hsdR514 ΔaraBAD$_{AH33}$ ΔrhaBAD$_{LD78}$*	[6]
pWKS30	*P$_{lac}$ polylinker Am^r*	[42]
pLEUCD2	*pWKS30 leuCD+ insert*	This study
pCKR101	*P$_{lac}$–lacI ^{q} P$_{tac}$ polylinker Am^r*	Jeff Gardner

Table 2.1 (cont.)

pLEUCD3	*pCKR101 leuCD+ insert*	This study
pFUMA	*pCKR101 fumA+ insert*	This study
pCP20	*FLP expression plasmid; amp, temperature-sensitive replication and FLP synthesis*	[5]
pKD3	*Template plasmid; amp, FRT-flanked cat*	[6]
pKD46	*λ Red recombinase (γ,β, and exo) expression plasmid; amp, ara-inducible expression, temperature-sensitive replication*	[6]

2.6 FIGURES

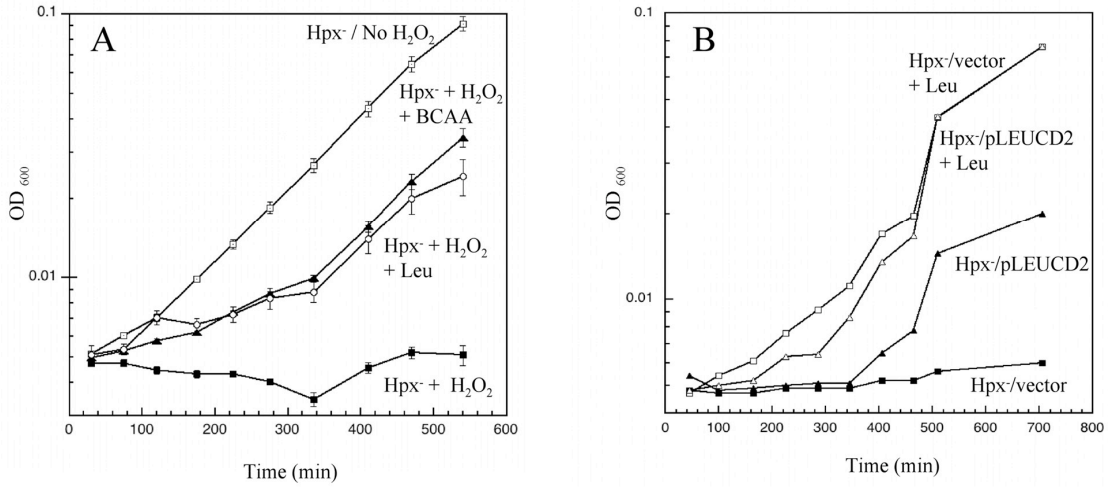

Figure 2.1 Low concentrations of H_2O_2 block leucine biosynthesis. (A) Hpx^- cells were cultured in aerobic glucose medium. Where indicated, 8 μM H_2O_2, branched-chain amino acids (BCAA: Leu, Ile, and Val), and/or leucine were added. (B) Hpx^- cells containing either pLEUCD2 (overproducing isopropylmalate isomerase) or pWKS30 (an empty vector) were cultured in lactose minimal medium with or without leucine supplementation. All media included histidine and aromatic amino acids. The pLEUCD2 plasmid boosted the isopropylmalate isomerase activity of anaerobic cultures about three-fold.

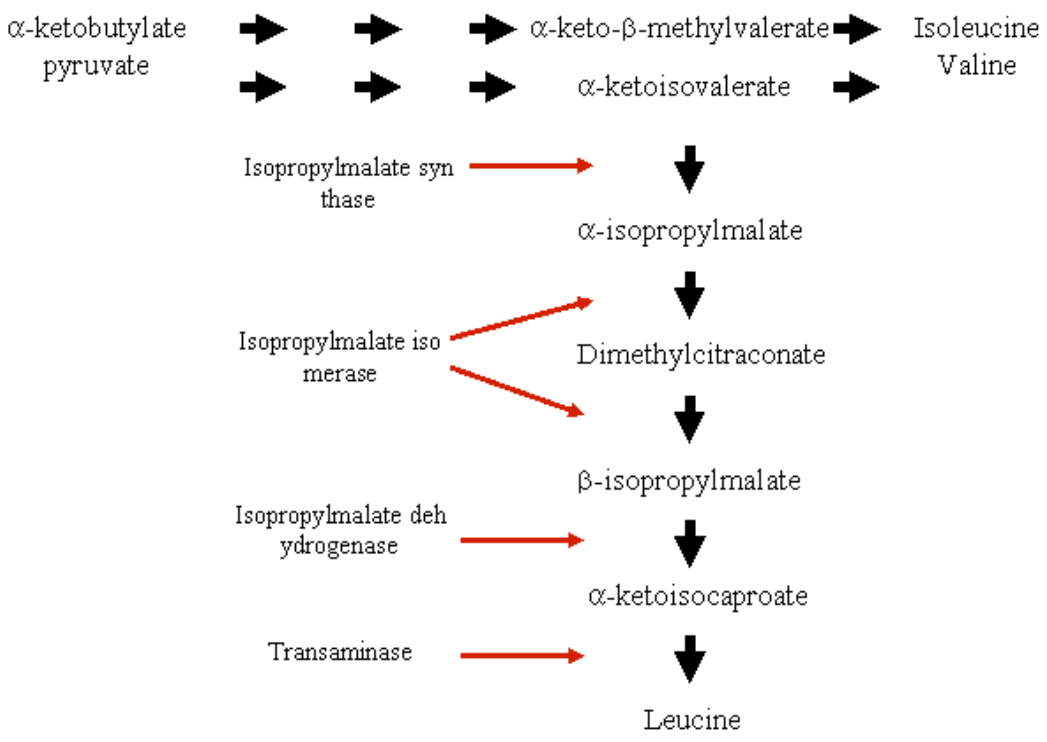

Figure 2.2 The branched-chain amino acid biosynthetic pathway.

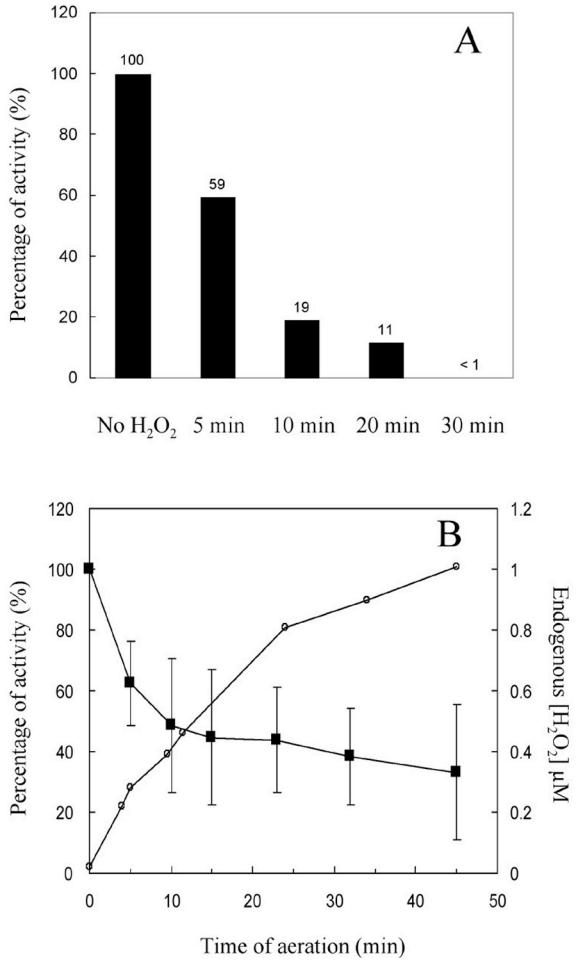

Figure 2.3 H_2O_2 rapidly inactivates isopropylmalate isomerase in vivo. (A) Hpx^- cells were anaerobically cultured to an OD_{600} of 0.2, and 8 μM H_2O_2 was then added to the cultures. At intervals catalase was added and residual enzyme activity was determined. (B) Hpx^- cells were cultured in anaerobic medium and then aerated starting at time zero. Isopropylmalate isomerase activity (squares) and H_2O_2 concentrations (circles) were monitored.

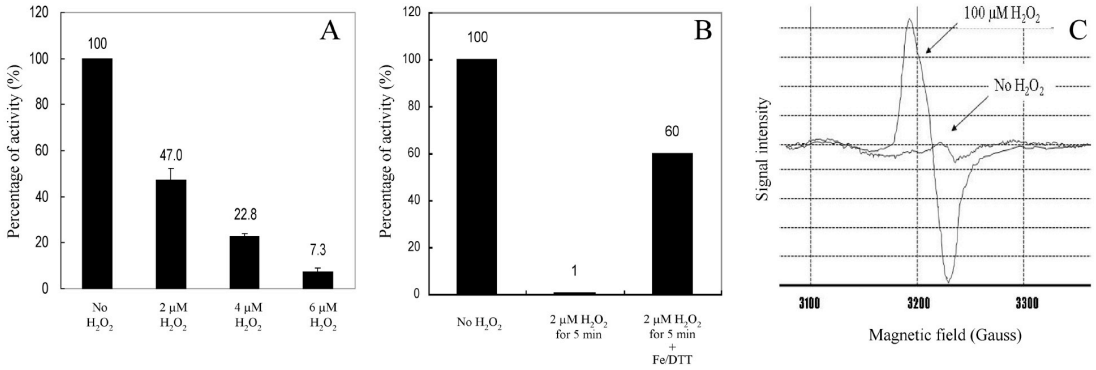

Figure 2.4 H_2O_2 inactivates isopropylmalate isomerase by converting its $[4Fe\text{-}4S]^{2+}$ cluster to a $[3Fe\text{-}4S]^{+}$ cluster in vitro. (A) An extract of anaerobically grown Hpx^- cells was exposed to the indicated concentrations of H_2O_2 for 2 min at room temperature. Catalase then was added to remove H_2O_2. The experiment was conducted anaerobically. (B) Isopropylmalate isomerase from anaerobically cultured Hpx^- cells was inactivated with 2 μM H_2O_2 for 5 min. Catalase was added to remove H_2O_2, and then 50 μM $Fe(NH_4)_2(SO_4)_2$ and 2.5 mM dithiothreitol were added. The activity was measured after 3 min incubation. (C) Hpx^- cells that overproduce IPMI were harvested when the cells were OD_{600} of 0.2. The cell pellets were resuspended in 1/500 of original culture volume of 10% glycerol. The resuspended cells were incubated with 100 μM H_2O_2 for 1 min at 37°C. The cell suspension (250 μl) then was transferred into an EPR tube and frozen.

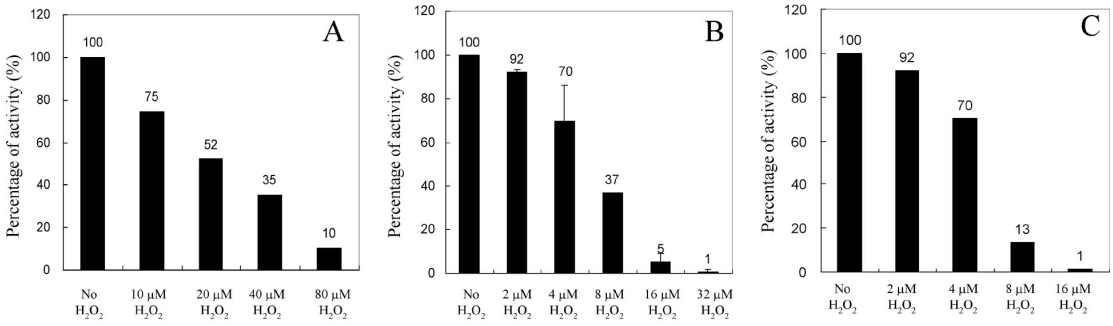

Figure 2.5 H_2O_2 inactivates other [4Fe-4S] dehydratases. (A) 6-phosphogluconate dehydratase. (B) Fumarase A. (C) Fumarase B. Lysates were prepared from anaerobic cultures of LC106, SJ37, and SJ20, respectively, and the indicated concentrations of H_2O_2 were added for 5 min. Catalase was added to terminate the stress, and residual activity was determined.

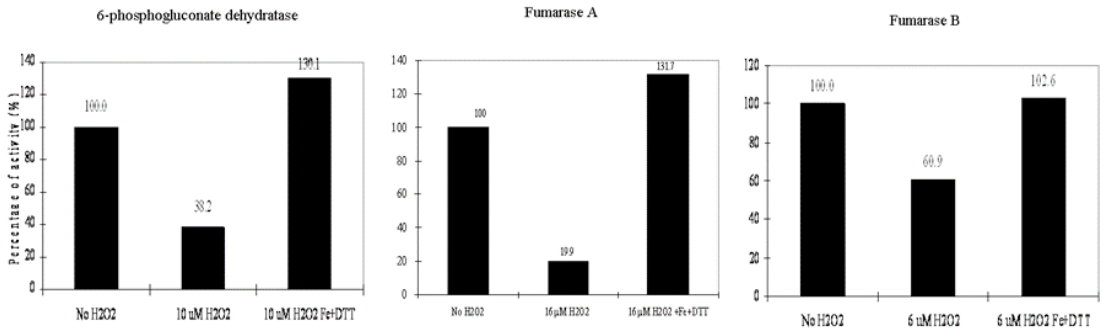

Figure 2.6 H_2O_2 inactivates [4Fe-4S] clusters in the active sites of dehydratases. Lysates were prepared from anaerobic cultures of LC106, SJ37, and SJ20, respectively, and the indicated concentrations of H_2O_2 were added for 5 min. Catalase was added to terminate the stress, and residual activities were measured. To repair inactivated enzymes, lysates were incubated with 400 μM $Fe(NH_4)_2(SO4)_2$/ 2.5 mM dithiothreitol for 10 min at room temperature.

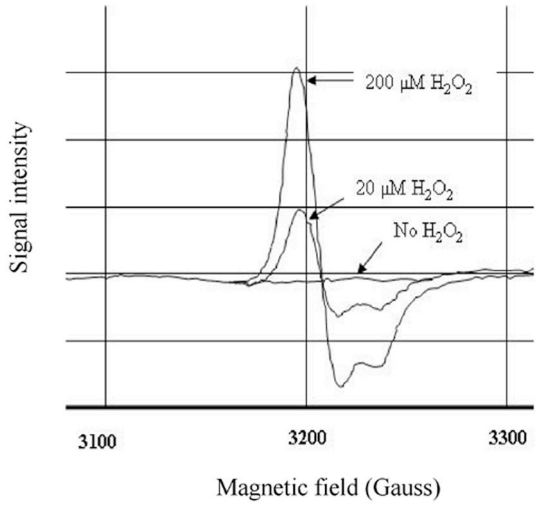

Figure 2.7 H_2O_2 inactivates fumarase A by converting its $[4Fe\text{-}4S]^{2+}$ cluster to a $[3Fe\text{-}4S]^{+}$ cluster in vivo. SJ37 cells that overproduce fumarase A were harvested at an OD_{600} of 0.2, washed, and resuspended at 1/500 of original culture volume in 10 % glycerol. The resuspended cells were incubated with 200 or 20 μM H_2O_2 for 1 min at 37°C. The cell suspension (250 μl) then was transferred into an EPR tube and frozen.

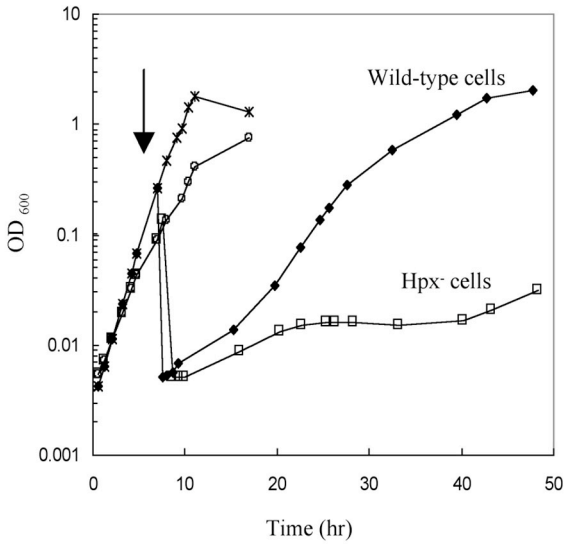

Figure 2.8 The Hpx$^-$ mutant is defective at using malate as the sole carbon source. Wild-type (stars) and Hpx$^-$ (open circles) cells were cultured in aerobic glucose medium containing to 0.15 – 0.25 (OD_{600}). At the arrow aliquots were removed, washed, and resuspended in aerobic malate medium. All media included histidine and aromatic amino acids.

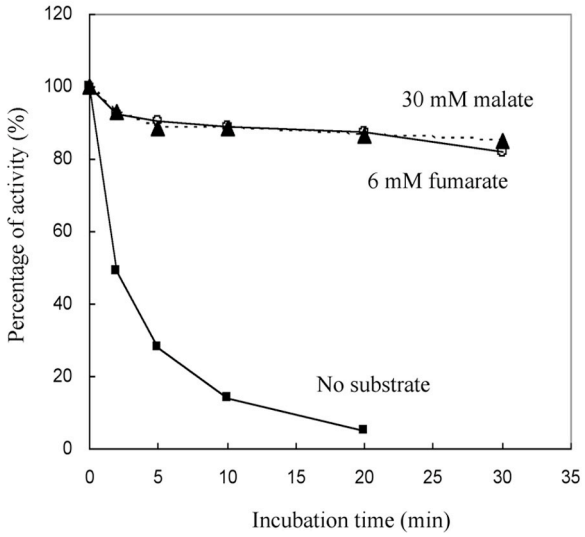

Figure 2.9 Substrates protect fumarase against H_2O_2. Purified fumarase A (5 nM) was exposed to 1 μM H_2O_2 at 0 °C. Where indicated 30 mM malate or 6 mM fumarate were included in the buffer. At time points, aliquots were removed, catalase was added, and residual activity was measured.

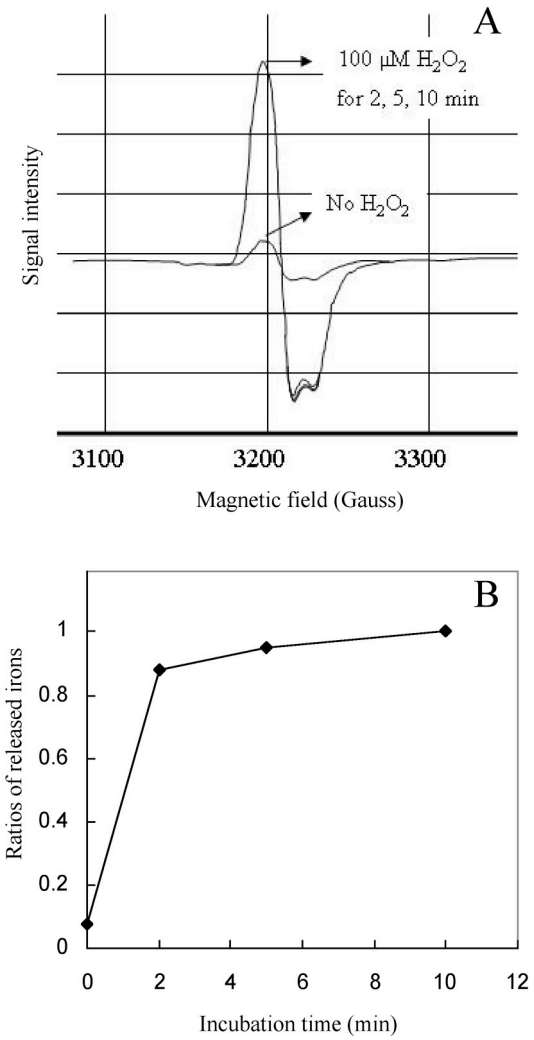

Figure 2.10 H_2O_2 degrades the $[4Fe\text{-}4S]^{2+}$ cluster of purified fumarase A to a $[3Fe\text{-}4S]^{+}$ cluster with loss of one iron atom. Purified fumarase A (18 μM) was inactivated by 100 μM H_2O_2 in 50 mM Tris-Cl/10 mM Mg^{2+} containing 1 mM desferrioxamine. At time points, aliquots were removed, catalase was added, and the reaction mixture was frozen. The residual cluster (panel A) and released ferric iron (panel B) were analyzed by EPR spectroscopy.

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CHAPTER 3: HYDROGEN PEROXIDE POISONS THE *ESCHERICHIA COLI* ISC CLUSTER ASSEMBLY SYSTEM, AND THE SUF SYSTEM IS INDUCED TO COMPENSATE

3.1 INTRODUCTION

Contemporary organisms inherited their biochemical mechanisms and metabolic pathways from ancestors that evolved in an anaerobic world. This creates problems when this machinery is employed in aerobic habitats. Inside cells molecular oxygen steals electrons from redox enzymes and continuously generates superoxide and hydrogen peroxide; these oxygen species are reactive and, therefore, potentially toxic. Organisms that live in aerobic habitats protect themselves from endogenous H_2O_2 by scavenging it with catalases and peroxidases. The titers of these enzymes are high enough to drive steady-state levels of H_2O_2 to levels as low as 20 nM [55].

Nevertheless, because H_2O_2 can cross membranes, microbes are rapidly toxified if its extracellular concentration is high. This situation can arise from abiotic processes, such as the oxidation of environmental sulfur or metals. It also occurs when organisms attempt to poison competitors by exploiting their vulnerability to reactive oxygen species. For example, lactic acid bacteria excrete H_2O_2 that inhibits the growth of other microbes; many plants and bacteria secrete redox-cycling antibiotics, which enter competitors and generate inhibitory oxidants; and mammalian macrophages deploy NADPH oxidase, which blasts captive bacteria with superoxide and H_2O_2 [31].

To understand oxidative stress we must identify the target biomolecules that are most vulnerable to it. Early work showed that millimolar doses of extracellular H_2O_2 are

moderately bacteriocidal, while micromolar doses are bacteriostatic [29]. The identification of the damaged biomolecules was thwarted by the robust scavenging activities of cells, which quickly degrade the H_2O_2 that is added to lab media and thus interfere with the imposition of chronic micromolar H_2O_2 stress. To circumvent this problem, one can analyze the injuries that accumulate in mutant strains that are devoid of scavenging enzymes. A mutant strain of *E. coli* that lacks both its catalases and its NADH peroxidase (*katE katG ahpCF*, denoted Hpx^-) does not degrade H_2O_2 at a significant rate [56]. When this strain is transferred from anaerobic to aerobic medium, endogenous H_2O_2 accumulates up to 1 micromolar. Since the H_2O_2 equilibrates between the intracellular and extracellular environments, external measurements allow intracellular concentrations to be tracked. Experiments with this strain have revealed several types of cell damage that occur rapidly. The Fenton reaction between H_2O_2 and unincorporated intracellular iron [reaction1] generates hydroxyl radicals, which oxidize a variety of biomolecules but exert their greatest toxicity by damaging DNA [27, 30, 39, 48].

(1) $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$$

H_2O_2 impedes the function of the Fur regulatory protein, apparently by oxidizing its iron cofactor; the consequence is a disruption of iron homeostatic mechanisms [67]. H_2O_2 also creates covalent polypeptide damage by reacting with the iron cofactors of

metalloproteins [2]. Finally, H_2O_2 oxidizes the solvent-exposed $[4Fe\text{-}4S]^{2+}$ clusters of a family of dehydratases [21, 33].

(2) $\quad Enz\text{-}[4Fe\text{-}4S]^{2+} + H_2O_2 \rightarrow Enz\text{-}[3Fe\text{-}4S]^{+} + 2OH^{-} + Fe^{3+}$

The catalytic iron atom is released, thereby obviating enzyme activity. A residual $[3Fe\text{-}4S]^{+}$ cluster remains, and it is subsequently reactivated to the $[4Fe\text{-}4S]^{2+}$ form inside cells, through a process that has not been resolved. It is striking that all these mechanisms of cell damage derive from reactions between H_2O_2 and iron.

These are the targets that cells must protect when they enter H_2O_2-containing environments. To do so, a H_2O_2-responsive regulon is activated. In *E. coli* this regulon is controlled by the OxyR protein, which responds to as little as 0.2 micromolar intracellular H_2O_2 [12, 72]. This value appears to define a threshold concentration of H_2O_2 that is potentially toxic.

Genes that respond to OxyR should, therefore, comprise key defenses against H_2O_2; indeed, although Hpx^- mutants can grow in aerobic medium, Hpx^- *oxyR* mutants cannot [48]. Some of the OxyR-controlled defenses have been identified. Catalase (encoded by *katG*) and NADH peroxidase (*ahpCF*) are induced in order to drive down the level of H_2O_2 [56]. Dps, a ferritin-like protein, is induced to sequester loose iron; by doing so, it greatly suppresses Fenton-mediated damage to DNA and to proteins [24, 28, 48]. Fur is induced in order to restore repression of iron-import proteins [67]. A manganese importer (*mntH*) protects simple metalloproteins by replacing their iron cofactors with an alternative metal that will not react with H_2O_2 [2].

The *suf* operon is also induced [72]. The SufABCDE proteins comprise an iron-sulfur-cluster assembly system. Under routine growth conditions, the Suf system is not synthesized, as clusters are assembled by the housekeeping Isc system [7, 8, 62]. Therefore, it seemed plausible that the Suf system is induced during H_2O_2 stress in order to repair damaged iron-sulfur clusters, in distinction to their de novo synthesis by the Isc apparatus. Experiments in some bacteria (but not others) [54, 65] indicated that Suf enhanced their resistance to a bolus of millimolar H_2O_2. Most strikingly, mutants of *Erwinia chrysanthemi* that lacked the *suf* operon exhibited hypersensitivity to paraquat, a generator of intracellular superoxide and H_2O_2 [43]. Subsequent work showed that the activities of oxidant-sensitive dehydratases were reduced [44].

The availability of Hpx^- mutants enabled us to test whether Suf is important in protecting *E. coli* from physiological, sub-micromolar levels of H_2O_2. Further, it allowed us to address the question of why Isc alone is inadequate. We considered several possibilities: that Suf is designed specifically to repair damaged clusters; that Suf is dedicated to cluster assembly for a protein(s) that is exclusively used during H_2O_2 stress; or that Isc is inoperative in H_2O_2-stressed cells. We determined that sub-micromolar H_2O_2 disables the Isc system and that Suf assumes the role of both de novo synthesis and cluster repair.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Glycerol and D-glucose were obtained from Fisher Scientific; ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and sodium dithionite,

from Fluka; Amplex Red, from Invitrogen; His Gravitrap, from GE Healthcare; and ECL™ Western Blotting Detection Reagents, from Amersham. Sigma was the source of L-amino acids, bovine liver catalase, horse heart cytochrome c, rabbit muscle lactate dehydrogenase, chloramphenicol, ferric chloride, ferrous ammonium sulfate, 2,2′-dipyridyl, citraconate, disodium L(-) malic acid, D,L-dithiothreitol, hydrogen peroxide, isopropyl β-D-thiogalactopyranoside, β-lactose, NADH, deamino NADH (nicotinamide hypoxanthine dinucleotide, reduced form), trichloacetic acid, N, N-dimethyl-p-phenylenediamine, potassium D-gluconate, glutathione (reduced and oxidized), sodium sulfide, succinic acid, tri(cyclohexylammonium) 6-phosphogluconic acid, plumbagin, thiamine, and o-nitrophenyl- β-D-galactopyraniside, DEAE-sepharose (DFF: Fast flow and DCL-6B), horse radish peroxidase-conjugated anti-rabbit goat immunoglobulin G. Anti-*A. vinelandii* IscU antibody was a kind gift of Dr. Dennis R. Dean.

3.2.2 Bacterial strains

The strains and plasmids used in this study are listed in Table 3.1. Null mutations were created by the Red/Gam recombination [18], and confirmed by either PCR analysis or enzyme assays. Mutations were introduced into new strains by P1 transduction and selected on media containing appropriate antibiotics. All strains in the Hpx^- background were constructed anaerobically to avoid an outgrowth of suppressors, which may occur aerobically.

3.2.3 Growth conditions

Anaerobic cultures were grown in an anaerobic glove box (Coy Laboratory Products Inc.), and aerobic cultures were grown with vigorous shaking in a water bath at

37°C. Standard minimal medium contained minimal A salts, 0.2% glucose, 1 mM $MgCl_2$, 5 mg/liter thiamine, and 0.5 mM histidine. Histidine was always added to the media because the parent strain, MG1655, is a histidine auxotroph under anaerobic conditions; to minimize the difference between anaerobic and aerobic cultures, histidine was also added to aerobic cultures. Casamino acids or specified amino acids were present at 0.2 % or 0.5 mM, respectively.

To ensure that Hpx^- derivatives were growing exponentially before they were exposed to an aerobic medium, anaerobic overnight cultures were diluted to OD_{600} = 0.005 in appropriate fresh anaerobic media and grown to OD_{600} ~ 0.1 prior to dilution into aerobic media.

3.2.4 Enzyme assays

For assays of labile [4Fe-4S]-cluster-containing enzymes--isopropylmalate isomerase (IPMI), fumarase, and 6-phosphogluconate dehydratase--cell extracts were prepared by sonication in an anerobic chamber using anaerobic buffers. IPMI activity was measured by monitoring the decrease of citraconate, a pseudo-substrate, at 235 nm in 100 mM Tris-Cl (pH 7.6). The fumarase activity was determined in 50 mM sodium phosphate (pH 7.4) containing 50 mM of $_L$-malate; production of fumarate was monitored at 250 nm. To assay 6-phosphogluconate dehydratase, lysates were prepared from cultures grown in a minimal medium containing 0.2 % gluconate. The production of pyruvate by 6-phosphogluconate dehydratase (in 50 mM Tris-Cl, pH 7.65) was determined in the second reaction catalyzed by lactate dehydrogenase [22].

Inverted membrane vesicles were prepared for NADH dehydrogenase I (Ndh1) and succinate dehydrogenase (Sdh) assays. The activities were measured by monitoring

either deamino NADH (nicotinamide hypoxanthine dinucleotide, reduced form) oxidation by Ndh1 at 340 nm or cytochrome c reduction for Sdh at 550 nm in 50 mM aerobic potassium phosphate buffer (pH 7.8).

The desulfurase activity of IscS was assayed anaerobically by measuring sulfide production as described before with modifications[59]. Purified IscS was incubated with 2.5 mM cysteine and 5 mM dithiothreitol at 37°C for 5 min, and then the reaction was stopped by addition of 20 % trichloracetic acid. Precipitated IscS was removed by centrifugation, and the supernatant was incubated with 2 mM N, N-dimethyl-p-phenylenediamine (DPD) and 3 mM $FeCl_3$ at ambient temperature for 30 min in the dark. The concentration of methylene blue that results from the reaction of DPD and sulfide was then measured at 670 nm.

As a reporter enzyme for transcriptional fusions, β-galactosidase was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The final product, ortho-nitrophenol, was detected at 420 nm.

H_2O_2 concentrations in culture media were determined by the Amplex Red/horseradish peroxidase method[55]. Because H_2O_2 rapidly equilibrates across the membranes of cells that lack scavenging activities, the extracellular H_2O_2 concentration is an excellent approximation of the intracellular concentration.

3.2.5 Plasmid constructions

Open reading frames of desired genes were PCR-amplified from *E. coli* MG1655 by using the primers in Table 3.2. The PCR products were digested with XbaI and EcoRI and cloned into either pWKS30 or pCKR101 vectors. The plasmid constructions were confirmed by restriction/sequencing analyses. In addition, complementation experiments

were performed in the Hpx^+ backgrounds to verify that the plasmids were functional. The proteins encoded by pWKS30 or pCKR101 derivatives were expressed in lactose or glucose/1 mM IPTG media, respectively.

3.2.6 Construction of transcriptional fusions

Single-copy chromosomal transcriptional fusions were constructed by the CRIM plasmid-host system [26]. Promoter regions of genes were PCR-amplified with primers in Table 2 and inserted into pSJ501 at KpnI and PstI restriction sites. The plasmids that contain transcriptional fusions were then integrated at the *attB* site (att_λ) on chromosomal DNA by the integrase that is provided by pAH57. Single-copy integrants were verified by PCR. The transcriptional fusions in chromosomal DNA were introduced into desired strains by P1 transduction. Unless otherwise indicated, each fusion strain retained a working wild-type copy of the gene at its normal locus.

3.2.7 Inactivation and reactivation of enzymes

Cells were grown anaerobically for at least four generations to $OD_{600} = 0.2$. H_2O_2 was added to the cultures when they were aerated. At time points, aliquots were removed, catalase was added to 200 U/ml, and cells were returned to the anaerobic chamber for centrifugation, lysis, and assay.

In vivo inactivation of IPMI by endogenous H_2O_2 was initiated by aerating anaerobic Hpx^- cultures without any addition of exogenous H_2O_2. Under these conditions the cells steadily generate H_2O_2, which was measured to be 0.5 – 1 μM. Cell extracts were then prepared and assayed in the anaerobic chamber.

The inactivation of enzymes in vitro was accomplished by the addition of H_2O_2 to lysates or to purified enzyme in anaerobic buffer. The H_2O_2 was subsequently removed by catalase prior to anaerobic assay. In some cases, damaged [3Fe-4S] clusters were chemically repaired by incubation with 100 μM $Fe(NH_4)_2(SO_4)_2$ and 2.5 mM dithiothreitol (DTT) for at least 10 min at room temperature. In vitro reconstitution of more extensively degraded iron-sulfur clusters was carried out by incubating enzymes in 50 mM Tris-Cl (pH 7.65) containing 500 μM $Fe(NH_4)_2(SO_4)_2$, 5 mM DTT, 2.5 mM cysteine, and purified IscS (0.16 mU) at room temperature for 20 - 60 min.

3.2.8 Purification of enzymes

Amplified *iscS* and *fdx* were inserted in pET15b at NdeI and BamHI restriction sites. *E. coli* BL21(DE3) cells containing pIsc-$(His)_6$ were grown aerobically in LB to OD_{600} of approximately 0.1, and 1 mM IPTG was added, followed by another four-hour incubation at 37°C. The overexpressed IscS-$(His)_6$ was purified using His Gravitrap. To avoid contamination of catalases, Fdx was overexpressed in a catalase mutant, JI367. JI367 was co-transformed with pFdx-$(His)_6$ and pTara that expresses T7 polymerase. T7 polymerase was induced in LB containing 0.1 % glucose and 0.25 % arabinose. The overexpressed Fdx-$(His)_6$ was purified using His Gravitrap.

Ferredoxin was also purified without a His tag. The Hpx^- strain that contains pTrcfdx was grown anaerobically to OD_{600} ~ 0.1 at 37°C, and 1 mM IPTG was added. To achieve the optimum synthesis of ferredoxin, the cells were grown another 12 hours at room temperature. The cells were harvested by centrifugation, and the cell pellets were resuspended in 15 ml of anaerobic 50 mM Tris-Cl/10 mM β–mercaptoethanol/0.1 mM EDTA (TBME, pH 7.4). Cell lysates were prepared by sonication, and cell debris was

removed by centrifugation. The purification was performed as described before with modifications [61]. All steps of the purification were conducted in an anaerobic chamber at room temperature, and all buffers were anaerobic. The cell extract was loaded onto a DEAE-Sepharose column (DFF: Fast flow) and eluted by 0.2 M of KCl in TBME. The fractions that showed the visible spectrum of $[2Fe\text{-}2S]^{2+}$ clusters were pooled and then loaded onto the second DEAE column (DCL-6B). The proteins were eluted by 0 – 0.4 M gradient of KCl in TBME. The purified ferredoxin was concentrated using Amicon ultra-15 centrifugal filter (3 kDa cutoff). The fractions that showed the spectrum of $[2Fe\text{-}2S]^{2+}$ clusters were frozen in dry ice containing ethanol. Purified ferredoxin was more than 90% pure based on SDS-PAGE and was quantified by dye-binding assay using bovine serum albumin as a standard.

Fumarase A was overproduced and purified as described previously [33].

3.2.9 Western blot analysis

Cells were grown for at least four generations anaerobically to an OD_{600} of approximately 0.1. Aerobic growth was then initiated by vigorous aeration of the cultures. At time points cells were harvested, and samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Anaerobic cultures were also harvested as controls. Samples that contained 42 μg of proteins were loaded on 12 % SDS-PAGE gel. Gels were either stained with Coomassie blue dye or transferred to nitrocellulose. After blotting with a 5% skim milk solution, a 1:1,000 dilution of rabbit serum containing anti-*A. vinelandii* IscU was used as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit goat immunoglobulin G was

used as the secondary antiserum (1:20,000 dilution). The detection process was performed using chemiluminescent reagents (ECL™ Western Blotting Detection Reagents).

3.2.10 EPR analysis of Isc proteins

Hpx$^-$ cells expressing genes of the *isc* operon behind the *tac* promoter were grown in minimal glucose medium anaerobically, and 1 mM IPTG was added when cells were at an OD_{600} of 0.2. After another 4 hours of incubation, the cells were harvested by centrifugation at 7500 rpm for 10 min, and the cell pellets were resuspended in 1/500 of original culture volumes of 10 % glycerol. The resuspended cells were incubated with H_2O_2 for 5 min at room temperature, and then catalase was added to remove H_2O_2. In the attempts to detect [2Fe-2S] or [4Fe-4S] clusters, 10 mM sodium dithionite was added. The cell suspension (250 µl) then was transferred into an EPR tube and frozen in dry ice.

EPR spectra of [Fe-S] clusters were obtained with the following settings: microwave power, 1 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 8 Gauss at 100 KHz; time constant, 0.032; temperature, 15 K.

3.2.11 Intracellular free-iron measurements

Cells were grown in minimal glucose medium to an OD_{600} of 0.1-0.2 and harvested by centrifugation at 7000 g for 5 min at 4ºC. A cell pellet was resuspended in 8 ml prewarmed fresh minimal medium that contain 10 mM DETAPAC (diethylentriaminepentaacetic acid, pH 7.0) and 20 mM desferrioxamine (pH 8.0). DETAPAC blocks further iron import, while desferrioxamine diffuses into cells and binds unincorporated iron in an EPR-visible ferric form. The concentrated cells were incubated

at 37 ºC for 15 min shaking in a water bath. The cells were washed with 5 ml of ice cold 20 mM Tris-Cl (pH 7.4) twice. Cells were then resuspended in 200 µl of ice cold 10% glycerol/20mM Tris-Cl (pH 7.4). The cell suspension (200 µl) then was transferred into an EPR tube and frozen in dry ice. Ferric sulfate standards were mixed with desferrioxamine and prepared in the same Tris buffer containing glycerol. The spectrometer settings were as follows: microwave power, 10 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; temperature, 15 K.

3.3 RESULTS

3.3.1 The Suf system is critical during H_2O_2 stress

Previous studies have shown that when *E. coli* is grown in standard lab media the Isc system constitutes the major mechanism of [Fe-S] cluster assembly [7, 8]. In agreement with that conclusion, we observed that *ΔhscA* mutants grew substantially more slowly in a minimal glucose medium (t_D = 89') than did the wild-type parent strain (t_D = 69'). Moreover, the enzymatic activities of two [Fe-S]-dependent enzymes, isopropylmalate isomerase and NADH dehydrogenase I, were reduced to 25% and 10% of their wild-type value. (For this purpose *ΔhscA* mutants were studied because they lack Isc function but retain IscS-dependent activities that are unrelated to cluster assembly.) The residual activities of these enzymes were apparently due to the action of the Suf system, which is induced in *Δisc* mutants by apo-IscR (Fig. 3.1). Double *Δisc suf* mutants cannot build clusters at all and are inviable [63].

In contrast, deletions of the *suf* operon caused no growth defect (t_D = 69'), nor were enzyme activities detectably diminished. Thus, as others have inferred [20, 46], the Suf system has no apparent role under standard growth conditions.

The observation that *suf* is controlled by the OxyR [37, 72] protein prompted us to examine whether the Suf system becomes important when cells are exposed to H_2O_2. Other workers have noted recovery defects when *Δsuf* mutants were challenged with a bolus of millimolar H_2O_2 [43, 44, 65]. Because the OxyR system is activated by sub-micromolar concentrations of intracellular H_2O_2 [4], we sought to test the role of Suf when cells were chronically exposed to low, physiological doses. To do so we employed *Δahp katG katE* (Hpx^-) mutants, which lack catalase and peroxidase activities and therefore cannot scavenge H_2O_2. When these mutants are cultured in aerobic medium, they accumulate 0.5-1.0 micromolar H_2O_2, which is generated by the spontaneous oxidation of intracellular redox enzymes [55]. This H_2O_2 comprises a constant oxidative stress. Hpx^- mutants are able to grow under these conditions, but they display reduced activities of some [Fe-S] dehydratases because H_2O_2 oxidatively damages their solvent-exposed [Fe-S] clusters [33].

When anaerobic cultures of Hpx^- mutants were diluted into aerobic medium, the *suf* operon was expressed at a level 40-fold higher than in wild-type cells, consistent with its induction by the OxyR protein (Fig. 3.2). Hpx^- *Δsuf* mutants grew somewhat slower than did their suf^+ parent. The addition of 6 μM H_2O_2 completely abolished their growth of the Hpx^- *Δsuf* strain; under the same conditions the parent continued to grow well (Fig 3.3A). The growth defect of the Hpx^- *Δsuf* mutant was eliminated when the *suf* operon was expressed from a plasmid (Fig. 3.3B). An Hpx^- *suf (non-inducible)* mutant—which retained the intact *suf* operon but lacked the OxyR binding site upstream—exhibited a

growth rate intermediate between that of the Hpx$^-$ *Δsuf* mutant and the Hpx$^-$ *suf*$^+$ strain (data not shown). Thus the Suf system is critical for growth during H_2O_2 stress, and OxyR must induce it to achieve sufficient levels.

Interestingly, Hpx$^-$ *ΔhscA* mutants were able to grow efficiently in aerobic medium, even when the exogenous H_2O_2 was added. This contrasts with the problems displayed by *ΔhscA* mutants in an Hpx$^+$ background, and it suggests that the OxyR-driven induction of Suf might fully compensate for the absence of the Isc system.

Most [Fe-S] enzymes are dispensable for growth in glucose medium, but isopropylmalate isomerase (IPMI) is necessary for the synthesis of leucine. Indeed, leucine supplementation restored the growth of the Hpx$^-$ *Δsuf* mutant. IPMI is among the most sensitive of the dehydratases that can be inactivated by H_2O_2, and measurements showed that its activity diminished rapidly in both Hpx$^-$ Suf$^+$ and Hpx$^-$ *Δsuf* mutants during their first hour of growth in 6 μM H_2O_2 (Fig. 3.4). However, whereas the Hpx$^-$ *suf*$^+$ cells ultimately retained about 10-15% of their original IPMI activity, the Hpx$^-$ *Δsuf* mutants did not. After four hours of aeration, the latter strain lacked detectable activity. The absence of IPMI in the *Δsuf* background occurred despite a compensatory induction of *leuCD* transcription, which is responsive to the intracellular concentration of leucine (Fig. 3.5).

Fumarase A and 6-phosphogluconate dehydratase are also [Fe-S] dehydratases whose activities are vulnerable to H_2O_2 [33]. The activities of these enzymes were similarly lower in Hpx$^-$ *Δsuf* mutants than in Hpx$^-$ Suf$^+$ cells (Fig. 3.6). Therefore, we concluded that the Suf system is needed to preserve [Fe-S] dehydratase activities during low-grade H_2O_2 stress.

3.3.2 The Suf system is needed to repair [Fe-S] clusters that have degraded beyond the $[3Fe\text{-}4S]^{+}$ state

In principle Suf could enhance dehydratase activity in H_2O_2-stressed cells through either of two ways: by inserting clusters de novo into newly synthesized polypeptide, or by rebuilding clusters that had been oxidatively damaged by H_2O_2. Given the circumstances, we suspected that the Suf system facilitated cluster repair. To test this idea, we monitored the activity of dehydratases under conditions in which de novo enzyme synthesis was blocked. An Hpx^{-} *Δsuf* strain was constructed in which Suf was expressed from a plasmid under the control of the *lac* promoter; when this strain was cultured in lactose medium, the Suf system was synthesized, even in anaerobic conditions when it is typically not expressed. Both this strain and its Hpx^{-} *Δsuf* complement were grown to exponential phase in anaerobic lactose medium, and then chloramphenicol was added to block further protein synthesis. The cultures were then aerated, and the activity of IPMI was monitored during the subsequent period of H_2O_2 stress. The data in Fig. 3.7A show that the Suf system significantly decreased the rate at which H_2O_2 inactivated IPMI. Thus its protective effect was consistent with a repair activity that partially countervailed the damaging effects of H_2O_2.

The experiment was repeated with fumarase B. Since this enzyme is less sensitive to H_2O_2, 20 μM exogenous H_2O_2 was added to the aerobic medium. In contrast to IPMI, fumarase B activity was unaffected by the presence or absence of the Suf system (Fig. 3.7B). This discrepancy led us to wonder whether the damaged clusters of oxidized IPMI and fumarase B might be in different forms.

When H_2O_2 initially oxidizes a $[4Fe\text{-}4S]^{2+}$, it quickly loses its catalytic iron atom and is converted to a $[3Fe\text{-}4S]^{+}$ form. This cluster can be rapidly repaired to the original

$[4Fe\text{-}4S]^{2+}$ state by incubation in vitro with ferrous iron and dithiothreitol; full activity is restored. We have documented this behavior for both IPMI and fumarase [33]. However, the iron/dithiothreitol treatment is not expected to reactivate enzymes whose clusters have degraded beyond the $[3Fe\text{-}4S]^{+}$ state—for example, to [2Fe-2S] or apo-protein forms—as sulfur atoms must be supplied to rebuild the cluster [60, 66]. Based on this idea, we explored the status of the oxidized clusters in IPMI and fumarase. Figure 3.8 shows that iron/ dithiothreitol treatment completely restored the fumarase activity in extracts prepared from H_2O_2-exposed Hpx^{-} *Δsuf* cells. Thus all the inactive fumarase enzymes were in the [3Fe-4S] form. In contrast, the same treatment restored less than 20% of the IPMI activity. Full restoration was achieved only when purified IscS and cysteine were provided as sulfur donors, indicating that the majority of clusters were degraded beyond the [3Fe-4S] form.

The observation that IPMI clusters degraded in vivo contrasted with our previous studies [33], which showed that oxidation creates a $[3Fe\text{-}4S]^{+}$ form that is stable in vitro for 60 min. We reproduced that result, using EPR to monitor cluster status. Whole-cell EPR was then used to track the cluster status when IPMI-overproducing cells were exposed to H_2O_2. Initially, a strong $[3Fe\text{-}4S]^{+}$ signal appeared; in contrast to the in vitro experiments, however, the signal decayed within five minutes (Fig. 3.9). During this period no activity was restored, suggesting that the disappearance of the signal was due to degradation of the cluster to a lesser form. EPR analysis failed to detect [2Fe-2S] clusters, raising the prospect that the cluster was fully removed. The mechanism of intracellular IPMI cluster degradation was probed but not solved (Discussion).

These data suggested the reason that IPMI, but not fumarase, required the Suf system for cluster repair. The Isc and Suf systems assemble full clusters on scaffolds, and

they would therefore seem to be capable of transferring full clusters to client apoproteins; however, they would appear to be unsuitable for the reactivation of a protein that retains a partial cluster. In agreement with this idea, a recent study showed that the Isc system can reactivate apo-FNR [42]. We also observed that over-expression of the *isc* operon from a plasmid diminished the rate of IPMI inactivation by H_2O_2 (data not shown), suggesting that the Suf and Isc systems work in equivalent ways, albeit the Suf system might be more effective during H_2O_2 stress.

In contrast, reactivation of [3Fe-4S] enzymes appears not to require a scaffold system. When purified fumarase A was exposed to H_2O_2 in vitro, the resultant $[3Fe\text{-}4S]^+$ form of the enzyme was efficiently reactivated when it was added to cell extracts, whether or not they contained Suf proteins (Fig. 3.10). Fumarase A was also efficiently reactivated by *Δsuf* mutants in vivo (Fig. 3.11). The mechanism of reactivation presumably requires the consecutive donation of an electron donor and ferrous iron, but we have not identified any cell proteins that are essential. The di-iron protein YtfE has been suggested to be a possible partner in this process [34, 35], but under our experimental conditions the rate of IPMI inactivation was not different in Hpx^- and Hpx^- *ΔytfE* mutants, and there was no difference in the rates at which $[3Fe\text{-}4S]^+$ clusters of fumarase A were repaired in vivo after brief exposure to H_2O_2 (Fig. 3.12).

In summary, the Suf system is necessary for the repair of IPMI, because the oxidized IPMI cluster substantially degrades in vivo. In contrast, the $[3Fe\text{-}4S]^+$ form of the oxidized fumarase cluster is stable and can be repaired without the involvement of Suf.

3.3.3 In H_2O_2-stressed cells the Suf system is also required for the de novo synthesis of clusters, due to the poor function of the Isc system

Since the preceding data indicate that Suf is needed to build clusters in apo-IPMI, one might infer that the Isc system is poorly functional when *E. coli* is stressed by H_2O_2. To test this idea more directly, we examined the activation of enzymes which rely on [Fe-S] clusters but whose clusters are shielded from H_2O_2 and therefore do not require repair. NADH dehydrogenase I and succinate dehydrogenase contain nine and three iron-sulfur clusters, respectively [15, 45]. The clusters are buried in polypeptide, and so the enzymes did not lose activity when they were exposed to even 1 mM H_2O_2 in vivo or in vitro (Fig. 3.13). These respiratory enzymes are repressed in our anaerobic glucose medium, due to the action of ArcAB and Fnr [16, 25], but they are induced when *E. coli* cells are aerated (data not shown). Figure 3.14 demonstrates that the enzyme activities quickly rose when Hpx^- Suf^+ cells were transferred to aerobic medium, but not when Hpx^- *Δsuf* mutants were transferred, although there was no difference in the *nuo* transcription level between two strains. In addition, the defect in the latter cells was not due to a general problem with protein synthesis, since these enzymes are not requisite for growth in glucose medium and the Δ*suf* strain grew as well as its Suf^+ partner. Instead, the result indicates that the submicromolar (0.5-1 μM) H_2O_2 that accumulated inside cells during these experiments was sufficient to prevent the Isc system from inserting new clusters into nascent polypeptides. In contrast, the progressive induction of the Suf system by OxyR circumvented this block.

To double-check this result, we indirectly monitored the presence of [2Fe-2S] clusters in the IscR protein. When cluster assembly is defective, the accumulation of apo-IscR protein allows it to serve as a transcription factor that activates expression of the

suf operon [23, 38, 71]. A *lacZ* transcriptional fusion was constructed immediately downstream of the *suf* promoter, and the upstream OxyR binding site was removed, so that expression was primarily dictated by the status of IscR. This fusion was expressed at a two-fold higher level in an Hpx$^-$ strain relative to Hpx$^-$ *ΔiscR* mutants. A further two-fold induction of suf$^{NI\text{-}OxyR}$::lacZ was detected in Hpx$^-$ *Δsuf* mutants. These data confirm that H_2O_2 interferes with the activity of the Isc system (Fig. 3.15), and they show that the Suf system substantially compensates.

3.3.4 The Isc system is present but non-functional during H_2O_2 stress

Collectively, these data indicate that the Suf system is induced in H_2O_2-stressed cells because the Isc system is inactive. One explanation for its inactivity might be that its synthesis was repressed. A transcriptional fusion to the *iscSUA* promoter was constructed; the fusion retained the RyhB sRNA binding site to ensure that Fur-mediated control was active [19]. We observed that the expression of the Isc system was very low in anaerobic wild-type cells, and it rose rapidly when the cells were aerated. The same result was observed in Hpx$^-$ cells, indicating that the dysfunction of the Isc system was not due to a lack of expression (Fig. 3.16A). In fact *isc* expression was somewhat higher in the H_2O_2-stressed cells than in unstressed cells, presumably reflecting the inducing effect of apo-IscR. Western blot analysis using anti-IscU antibody verified the presence of this Isc protein in Hpx$^-$ cells (Fig. 3.16B). Thus H_2O_2 apparently inhibits the function of extant Isc system.

3.3.5 The inactivity of the Isc system is reversible

We sought to identify the mechanism by which H_2O_2 disrupts the Isc system. As a first step, we tested whether the system would regain function when H_2O_2 was removed. To do so, we took advantage of the observation that rapidly synthesized [Fe-S] proteins transiently accumulate in an apo-protein form, due to the insufficient synthesis of [Fe-S] clusters. The *fumA* gene was strongly induced from a *tac* promoter in aerobically grown wild-type and Hpx$^-$ *Δsuf* strains. Under these conditions ca. 80% of the protein accumulated in the apo-protein form. Chloramphenicol was added to block further fumarase and Isc synthesis. When aerobic incubation continued, the wild-type cells progressively activated fumarase; however, no significant increase in fumarase activity occurred in the Hpx$^-$ *Δsuf* strain, indicating that the accumulated H_2O_2 continued to inhibit the Isc system. In contrast, when cells were moved to anaerobic medium, fumarase activity rebounded in the Hpx$^-$ *Δsuf* strain at a rate comparable to that of wild-type cells (Fig. 3.17). Thus once H_2O_2 stress ceases, the Isc system regains activity. This reversibility may explain why *E. coli* continues to synthesize the Isc proteins even when inhibitory levels of H_2O_2 are present. It also constrains the possible mechanisms by which H_2O_2 blocks Isc function.

3.3.6 How does H_2O_2 poison the Isc system?

The Suf system is induced during iron starvation [46], which led to the observation that the Isc system works poorly when iron levels are low. During H_2O_2 stress the OxyR system works to minimize Fenton chemistry by reducing the levels of free iron in the cell. This is accomplished by induction of the Dps ferritin-like protein, which sequesters unincorporated iron, and by induction of the Fur repressor, which

diminishes synthesis of iron importers [48, 67]. Thus we explored the possibility that the effects of the OxyR-directed responses might starve the Isc system for iron.

Hpx$^-$ *ΔoxyR* mutants are not viable in aerobic medium [48], which precluded the most direct test of this notion; however, the OxyR system is also induced in *ahpCF* mutants, due to modestly elevated levels of intracellular H_2O_2 [55]. Assays showed that NADH dehydrogenase I was synthesized at normal rates in an *ahpCF suf* strain (data not shown). Further, an *oxyR2* expression plasmid that constitutively induces the OxyR response [36] also did not create either growth defects or [Fe-S] enzyme deficiencies in a *suf* background (Fig. 3.18). These data indicated that OxyR-controlled activities, including iron-depletion measures, are not sufficient to inactivate Isc. Indeed, EPR measurements, indicated that due to cluster damage the levels of unincorporated intracellular iron are actually higher in Hpx$^-$ mutants, in which Isc fails, than in wild-type cells, in which Isc is functional[67]. Finally, a cluster-synthesis defect could only be elicited in the *ΔahpF suf* strain when it was exposed to 20 μM exogenous H_2O_2; this defect was not diminished by the deletion of the *dps* gene (Fig. 3.19). Thus it appears to be H_2O_2 itself that poisons Isc function.

The Isc system consists of the IscS desulfurase, the IscU scaffold protein on which nascent clusters are assembled, an IscA protein that serves either in cluster transfer or iron delivery, a ferredoxin that might adjust the cluster redox state, and the HscA/HscB chaperone proteins that apparently assist in cluster transfer to client apoproteins [6-8]. To date only a few chemical effects of low-micromolar H_2O_2 have been detected in vivo; these include the oxidation of reactive cysteinyl residues and of solvent-exposed iron-

sulfur clusters [31]. The Isc system involves both, and either type of reaction would be consistent with the reversibility of Isc inactivation. Thus the potential targets were examined in turn.

The IscS desulfurase features an exposed active-site cysteine [17] that seemed to be a plausible target for oxidation. Most protein cysteine residues react far too sluggishly to be affected by micromolar H_2O_2, but the OxyR, AhpC, and OhrR proteins feature active-site cysteines that are activated by local context so they are easily oxidized to sulfenic acid [31]. However, we observed that purified IscS retained full activity when it was exposed to even 1 mM H_2O_2, whether the H_2O_2 was added in the presence or absence of cysteine (Fig. 3.20).

Uniquely among Isc/Suf proteins, IscA and SufA can substitute for one another in vivo : although *ΔiscA sufA* mutants are inviable, a *ΔsufA* mutant that lacks any of the Isc proteins other than IscA is viable, since the Suf system can function using IscA [40, 68]. We observed that Hpx$^-$ mutants exhibited growth defects if they additionally lacked SufBCD, but Hpx$^-$ *ΔsufA* mutants were healthy. The implication is that IscA is functional in H_2O_2-stressed cells. Thus the Isc dysfunction must arise from damage to a different Isc protein (Fig. 3.21).

The overexpression of the full *iscSUA-hscAB-fdx* gene set restored robust cluster synthesis in the Hpx$^-$ *Δsuf* mutants (Fig. 3.22); however, overexpression of individual genes failed to do so. Ferredoxin may dissociate during the catalytic cycle; if so, the fact that ferredoxin overproduction did not restore Isc function (Fig. 3.23) strongly suggests that it is not the limiting component in the H_2O_2-stressed system. Although we observed

that in vitro ferredoxin was rapidly oxidized by H_2O_2, the oxidized cluster was immediately reducible, indicating that the apparent Fenton chemistry did not generate disabling damage to the protein (data not shown).

Aside from IscA and ferredoxin, the other *isc* gene products may exist as part of a larger complex, so that overproduction of a single member cannot easily compensate for damage. Our suspicion was that the nascent iron-sulfur clusters that are built on IscU might be vulnerable to oxidative degradation in vivo, since they are likely to be solvent-exposed when they are transferred to recipient proteins. Further, workers have reported that clusters in IscU are degraded when they are exposed to aerobic buffers in vitro [1, 58]. To test this idea in vivo, we attempted to overproduce the IscSU complex in order to use whole-cell EPR to monitor the status of IscU clusters during exposure to H_2O_2. In agreement with a previous report, this effort was unsuccessful[52]; we were able to visualize clusters by EPR or visible spectroscopy only when the full *iscSUA-hscAB-fdx* operon was overexpressed. When these cells were exposed to 0.01 – 1 mM exogenous H_2O_2, no $[3Fe\text{-}4S]^+$ signal appeared. Treatment of the unstressed cells with dithionite generated a signal that was consistent with $[2Fe\text{-}2S]^+$ and/or $[4Fe\text{-}4S]^+$ clusters; previous treatment with as much as 1 mM H_2O_2 did not diminish the dithionite-generated signal, indicating that H_2O_2 did not destroy the clusters (Fig. 3.24). However, since the EPR analysis showed total [Fe-S] cluster spectrum of the Isc system, oxidation or degradation of the cluster on IscU might be obscured by the [2Fe-2S] signals of IscA and ferredoxin. Unfortunately, we were unable to make any definitive conclusion regarding the stability of IscU-bound clusters.

3.4 DISCUSSION

When Barras and his collegues exposed *E. chrysanthemi suf* mutants to phenazine methosulfate, a redox-cycling drug that generates reactive oxygen species, they discovered that dehydratase activities were very low [44]. Oxidants can damage dehydratase clusters [21, 33], and it seemed plausible that Suf was needed to repair them. But the observation was puzzling in two respects. First, why would a scaffold system be required to repair the [3Fe-4S] clusters that are produced by oxidation reactions? And second, in any case, why was the housekeeping Isc system inadequate for the job? The results from the present study begin to answer those questions. First, although damaged clusters are stable in vitro, some [3Fe-4S] clusters are further disassembled in vivo, thereby requiring the engagement of a de novo-style cluster-assembly system for their full repair. And second, the Isc system is poisoned by as little as 1 micromolar H_2O_2, leaving the apparently oxidant-resistant Suf system as the only viable Fe/S scaffold system. Thus during periods of oxidative stress bacteria rely upon the Suf system not only to maintain dehydratase activity but to support the activities of all Fe-S enzymes. Still, these results raise some intriguing questions.

3.4.1 How are damaged clusters disassembled in vivo?

Repair of [3Fe-4S] clusters can easily be managed in vitro by dithiothreitol and ferrous iron, and we imagine that an analogous process occurs in vivo. A dithiol is required for reactivation, which suggests that the iron-sulfur bonds in the cluster can reorganize themselves internally and can interact with solutes. This fact led us to wonder whether the degradation of the [3Fe-4S] IPMI cluster inside cells might occur through

sulfur-exchange reactions with metabolites. To test this notion, we attempted to replicate degradation by incubating the oxidized form of the enzyme in vitro with cysteine and with both oxidized and reduced glutathione; however, the $[3Fe\text{-}4S]^{+}$ cluster remained intact. Thus it seems likely that degradation is catalyzed by enzymes. The Suf system is not requisite, since disassembly occurs in *suf* mutants. At present the basis of IPMI cluster decay remains uncertain.

Interestingly, the [4Fe-4S] cluster of IRP-1—which controls iron levels in mammalian cells—appears to behave much like that of IPMI, with potentially important consequences for metabolism. Workers found that in vitro H_2O_2 treatment produces a [3Fe-4S] cluster containing IRP-1, which does not have both aconitase activity and its RNA binding activity [10]. However, upon oxidation in vivo the cluster seems fully degraded, thereby exposing an RNA binding site that interacts with the transcripts of iron-import and –storage proteins [11, 13]. Accumulation of the apo-protein form is normally a marker of iron starvation; during oxidative stress, however, the apo-protein form may inadvertently activate mechanisms that import more iron into the cell—thereby potentially aggravating the situation by stimulating Fenton chemistry. Aconitases have been proposed to serve similar iron-regulatory roles in *B. subtilis* and *E. coli* [64], and further experiments will be needed to determine whether oxidative stress affects them in a manner similar to IRP-1.

3.4.2 How does H_2O_2 poison the Isc system?

By refuting other possibilities, we inferred that H_2O_2 probably disrupts the Isc system by oxidizing the nascent clusters that are formed on the IscU scaffold protein. It seems particularly likely that the clusters are exposed to solvent—and to dissolved

oxidants—when they are transferred to recipient apoproteins. There is no easy way to test this idea biochemically, since H_2O_2 reacts rapidly with the ferrous iron that is used to build such clusters in vitro; this Fenton reaction will both scavenge the H_2O_2 and prevent catalytic turnover of the Isc system. Molecular oxygen does the same thing, forcing workers to use anaerobic conditions to study Isc function; thus, it is not instructive to demonstrate that H_2O_2 disrupts the system in vitro.

Electron transfer from iron to H_2O_2 is an inner-sphere process; that is, in order to react with iron, H_2O_2 must bind it directly. For this reason dehydratase clusters can be fully protected from H_2O_2 by bound substrates, which provide fifth and sixth ligands to the erstwhile solvent-exposed iron atom. The iron atoms in IscU clusters are comparatively undercoordinated, as each derive three ligands from bridging sulfur atoms and another either from a protein aspartate or cysteine residue; conceivably, they can be directly oxidized by species that would occupy a fifth coordination site. In fact, using UV-visible spectroscopy, other workers showed that H_2O_2 can directly oxidize the [2Fe-2S] clusters even in resting IscU protein [9]. However, in those experiments approximately millimolar H_2O_2 concentrations were used, which is a thousand times the concentration of H_2O_2 that we found was sufficient to inhibit Isc in vivo. Thus the physiological relevance of this mechanism of cluster oxidation is uncertain.

If Isc inactivation were to derive from cluster oxidation, it would fall in line with the other molecular injuries produced by H_2O_2, all of which arise from iron-oxidation reactions. This includes DNA damage [48], which is driven by a Fenton reaction between H_2O_2 and DNA-bound iron; dehydratase inactivation [21, 33]; disruption of Fur protein, in which H_2O_2 apparently oxidizes the ferrous iron cofactor [67]; and protein carbonylation, which also arises through Fenton chemistry [2]. No non-iron-related

injuries have been detected when bacteria are exposed to micromolar concentrations of H_2O_2. The rate constants for these reactions depend on the coordination environment of the iron atom but approximate 10^4 M^{-1} s^{-1} [33, 48]. The OxyR system is calibrated appropriately, responding when H_2O_2 levels exceed 0.2 micromolar [12, 31]—that is, at a dose above which the half-time of Fenton reactions approaches 1 min.

Since superoxide oxidizes dehydratase clusters at a rate even higher than does H_2O_2, we wondered whether superoxide might also be able to inactivate Isc. We found that a *suf::lacZ* fusion is substantially induced in SOD^- mutants. Further, 6-phosphogluconate dehydratase and succinate dehydrogenase activities were substantially reduced when a *suf* mutation was introduced into the SOD^- strain. The data indicate that superoxide, like H_2O_2, inhibits Isc function—which is consistent with an iron-sulfur target in the system. Indeed, the earlier observation that *suf* mutants are poisoned by phenazine methosulfate [44] might owe more to superoxide, which is the direct product of PMS cycling, than to H_2O_2.

3.4.3 Does the distribution of the Suf and Isc systems correlate with environmental stress?

These considerations suggest that the Suf system somehow shields its nascent clusters from small oxidants. Similarly, the Suf system is more resistant to soft metals [41, 51], which can disrupt solvent-exposed clusters, than is the Isc system. How Suf might shelter its clusters is entirely unclear. In any case, if the Suf system is more robust than the Isc system, one might guess that Isc is maintained in the biological world because it has its virtues, too, perhaps being catalytically more efficient than Suf. This

notion has not been tested, but it fits the fact that *E. coli* employs Isc as its housekeeping system and Suf only as an emergency back-up.

More generally, one might expect that organisms which routinely encounter oxidants might dispense with Isc and exclusively express Suf, whereas ones that do not encounter oxidants might do the opposite. A phylogenetic survey of the two systems gives mixed results. Lactic acid bacteria, which generate huge doses of H_2O_2 through their pyruvate and lactate oxidases, contain Suf and lack Isc. So do eukaryote pathogens such as *Mycobacterium tuberculosis, Enterococcus faecalis*, and *Xylella fastidiosa*, which need to withstand the H_2O_2 that is generated by the host immune response [5, 53]. Chloroplasts, which are substantial sources of reactive oxygen species, employ only the Suf system [3]. These data are consistent with the hypothesis. However, most anaerobic bacteria also have dispensed with the Isc system, relying instead on Suf or on the Nif system. Perhaps the Isc system is ill-suited for environments that are occasionally iron-limited. An alternative is that these microbes must periodically contend with the H_2O_2 that is chemically generated when aerated waters mix with anaerobic solutions of reduced sulfur and metals. Obligate anaerobes all express catalases and/or peroxidases [32], presumably for such a circumstance.

In contrast, non-photosynthetic eukaryotes are distinguished among organisms in that they rely entirely on Isc for cluster assembly—they do not have Suf even as a back-up system. This unique arrangement may be viable only because Fe-S assembly in eukaryotes occurs in the mitochondrial matrix and thus is shielded from the external environment by two membranes. Because membranes are only semi-permeable to H_2O_2, efficient scavenging systems are able to lower the cytoplasmic H_2O_2 concentration up to an order of magnitude lower than that outside the cell. Mitochondria have additional

scavengers [49, 50], so that second gradient would exist between the mitochondrial matrix and the cytosol. The effects of these layers of scavenging should be multiplicative, and it should rigorously protect the Isc system from external H_2O_2—apparently making the Suf system unnecessary. We note, though, that these diffusion barriers would not protect Isc from drugs like antimycin, which produces H_2O_2 within the mitochondria [47]. Such drugs potentially diminish the activities not only of oxidant-sensitive dehydratases but also of other Fe-S enzymes whose de novo activation would be impeded.

3.5 TABLES

Table 3.1 Strains and plasmids

Strains	Genotype and characteristics	Source or ref.
MG1655	F^- wild type *E. coli*	*E. coli* Genetic Stock Center
BW25113	*lacI rrnB ΔlacZ hsdK ΔaraBAD ΔrhaBAD*	[18]
OD114	MG1655 *ΔhscA2::cat zff-208::*Tn*10*	[20]
OD510	*Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10 sodA25*::Mu*dPR13 sodB1-Δ2*::*kan*	[20]
SJ89	MG1655 *Δ(sufABCDSE)19*::*kan~zdi57::Tn10*	P1(OD510) x MG1655
LC106	*ΔahpF::kan Δ(katG17::*Tn*10)1 Δ(katE12::*Tn*10)1*	[56]
SJ15	LC106 *Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10*	P1(SJ89) x LC106
SJ98	MG1655 *ΔlacZ1::cat*	This work
SJ100	LC106 *ΔlacZ1::cat*	P1(SJ98) x LC106
SJ102	SJ15 *ΔlacZ1::cat*	P1(SJ98) x SJ15
SJ130	MG1655 *Δ(lacZ1::cat)1*	This work
SJ108	LC106 *Δ(lacZ1::cat)1*	This work
SJ160	SJ15 *Δ(lacZ1::cat)1*	This work
SJ172	SJ130 *attλ::[pSJ501::sufA'-lac$^+$]~cat*	This work
SJ186	SJ108 *attλ::[pSJ501::sufA'-lac$^+$]~cat*	P1(SJ172) x SJ108
SJ57	LC106 *ΔhscA2::cat*	P1(OD114) x LC106
SJ283	BW25113 *Δ(OxyR binding site in PsufA)1::cat*	This work
SJ288	LC106 *Δ(OxyR binding site in PsufA)1::cat*	P1(SJ283) x LC106
SJ440	MG1655 *Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac$^+$]~cat*	This work
SJ442	LC106 *Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac$^+$]~cat*	P1(SJ440) x SJ108

Table 3.1 (cont.)

SJ445	SJ15 *Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac$^+$] ~cat*	P1(SJ440) x SJ160
SJ40	BW25113 *Δ(fumC::cat)1 ΔfumB1::cat*	This work
SJ53	LC106 *Δ(fumC::cat)1 Δ(fumB1::cat)1*	This work
SJ65	SJ53 *Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10*	P1(SJ89) x SJ53
SJ54	LC106 *Δ(fumCA::cat)1 Δ(fumB1::cat)1*	This work
SJ88	SJ54 *Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10*	P1(SJ89) x SJ54
SJ90	BW25113 *ΔytfE1::cat*	This work
JI366	*ΔkatG17::*Tn*10 Δ(katE12::*Tn*10)1*	[56]
SJ233	JI366 *ΔytfE1::cat*	P1(SJ90) x JI366
SJ91	SJ54 *ΔytfE1::cat*	P1(SJ90) x SJ54
SJ93	SJ88 *ΔytfE1::cat*	P1(SJ90) x SJ88
OD102	*hscA114*::*cat Φ(nuoAB-lacZ)*	[20]
SJ109	SJ100 *Φ(nuoAB-lacZ)*	λ (OD102) x SJ100
SJ110	SJ102 *Φ(nuoAB-lacZ)*	λ (OD102) x SJ102
λJS2	*Φ(sdhC-lacZ) lacY$^+$ lacA$^+$*	[57]
SJ150	MG1655 *Δfrd1::cat*	This work
SJ161	SJ108 *Δfrd1::cat*	SJ108 P1(SJ150)
SJ163	SJ160 *Δfrd1::cat*	P1(SJ150) x SJ160
SJ179	SJ161 *Φ(sdhC-lacZ)*	λ (λJS2) x SJ161
SJ220	SJ163 *Φ(sdhC-lacZ)*	λ (λJS2) x SJ163
SJ263	SJ130 *attλ::[pSJ501::sufA$^{'NI\text{-}OxyR}$-lac$^+$] ~cat*	This work

Table 3.1 (cont.)

SJ276	SJ108 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ~cat*	P1(SJ263) x SJ108
SJ334	SJ160 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ~cat*	P1(SJ263) x SJ160
SJ272	BW25113 *ΔiscR1::cat*	This work
SJ291	SJ130 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ΔiscR1::cat*	P1(SJ272) x SJ263
SJ293	SJ108 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ΔiscR1::cat*	P1(SJ272) x SJ276
SJ332	SJ263 *Δ(sufABCDSE)19::kan ~zdi57::*Tn*10*	P1(SJ89) x SJ263
KI232	*Δ(sodB::kan)1 ΔsodA-1*	Laboratory stock
SJ259	KI232 *Δ(lacZ1::cat)1*	P1(SJ98) x SJ297
SJ297	SJ259 *Δ(sufABCDSE)19::kan ~zdi57::*Tn*10*	P1(SJ89) x SJ259
SJ278	SJ259 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ~cat*	P1(SJ263) x SJ59
SJ295	SJ259 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ΔiscR1::cat*	P1(SJ272) x SJ278
SJ336	SJ297 *attλ::[pSJ501::sufA'$^{NI\text{-}OxyR}$-lac^{+}] ~cat*	P1(SJ263) x SJ297
SJ1010	SJ130 *attλ::[pSJ501::iscRS'-lac^{+}] ~cat*	This work
SJ1019	SJ108 *attλ::[pSJ501::iscRS'-lac^{+}] ~cat*	P1(SJ1010) x SJ108
SJ1021	SJ160 *attλ::[pSJ501::iscRS'-lac^{+}] ~cat*	P1(SJ1010) x SJ160
JI370	*ΔahpF::kan*	[56]
SP65	*ΔmhpC281*::Tn*10 lacY1 Δdps-1::cat*	[48]
SJ181	JI370 *Δdps-1::cat*	P1(SP65) x JI370
SJ183	SJ181 *Δ(sufABCDSE)19::kan ~zdi57::*Tn*10*	P1(SJ89) x SJ181

Table 3.1 (cont.)

SJ1001	BW25113 *ΔsufA1::cat*	This work
SJ1004	BW25113 *Δ(sufBCDSE)1::cat*	This work
SJ1023	BW25113 *Δ(sufSE)1::cat*	This work
OD112	*ΔiscA112*::*cat*	[18]
SJ1013	LC106 *ΔiscA112*::*cat*	P1(OD112) x LC106
SJ1015	LC106 *ΔsufA1::cat*	P1(SJ1001) x LC106
SJ1017	LC106 *Δ(sufBCDSE)1::cat*	P1(SJ1004) x LC106
SJ1042	LC106 *Δ(sufSE)1::cat*	P1(SJ1023) x LC106
SJ1046	LC106 *Δ(sufA1::cat)1 ΔiscA112*::*cat*	P1(OD112) x SJ1015
SJ424	BW25113 *Δbfr-1::cat*	This work
SJ448	BW25113 *Δ(bfd bfr)-1::cat*	This work
KCI540	LC106 *ΔcyaY1::cat*	Laboratory stock
JRG2953	W3110 *Δbfr::kan Δftn::spec*	J.R. Guest
SJ428	LC106 *Δbfr-1::cat*	P1(SJ424) x LC106
SJ432	SJ15 *Δbfr-1::cat*	P1(SJ424) x SJ15
SJ459	LC106 *Δ(bfd bfr)-1::cat*	P1(SJ448) x LC106
SJ453	LC106 *Δftn::spec*	P1(JRG2953) x LC106
SJ463	SJ15 *ΔcyaY1::cat*	P1(KCI540) x SJ15
SJ461	SJ453 *Δ(bfd bfr)-1::cat*	P1(SJ448) x SJ453
SJ466	SJ461 *Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10*	P1(SJ89) x SJ461

Table 3.1 (cont.)

SJ473	SJ453 *Δ((bfd bfr)-1::cat)1 ΔcyaY1::cat*	P1(KCI540) x SJ461
SJ487	SJ473 *Δ(sufABCDSE)19*::*kan~zdi57::*Tn*10*	P1(SJ89) x SJ473
Plasmid		
pCP20	FLP expression plasmid; Amp^r, temperature-sensitive replication and FLP synthesis	[14]
pKD3	Template plasmid; amp, FRT-flanked cat	[18]
pKD46	λ Red recombinase (γ,β, and *exo*) expression plasmid; amp, ara-inducible expression, temperature-sensitive replication	[18]
pCKR101	P_{lac}–*lacI* ^{q} P_{tac} polylinker Amp^r (20- 50 copies per cell)	Jeff Gardner
pWKS30	P_{lac} polylinker Amp^r (6 – 8 copies per cell)	[69]
pSUF1	pWKS30 containing *sufABCDSE*	This work
pISC1	pWKS30 containing *iscSUA hsc BhscA fdx iscX*	This work
pISC2	pCKR101 containing *iscSUA hscB hscA fdx iscX*	This work
pIscSUA	pWKS30 containing *iscSUA*	This work
pSJ104	pWKS30 containing *hscB hscA fdx iscX*	This work
pSJ107	pWKS30 containing *fdx iscX*	This work
pET15b	P_{T7} polylinker Amp^r	Lab stock
pIscS-$(His)_6$	pET15b containing *iscS*	This work
pFdx-$(His)_6$	pET15b containing *fdx*	This work
pTara	Pbad-T7 RNAP Cm^r	[70]
pTrcfdx	pTrc99 (P_{trc} polylinker Amp^r) containing *fdx*	[61]
pFUMA	pCKR101 containing *fumA*	[33]
pAH125	CRIM reporter plasmid (Km^r)	[26]
pSJ501	pAH125 derivative, in which the Kam resistant marker has been replaced with a *cat* gene.	This work
pAH57	CRIM helper plasmid	[26]
pACYC184	Tet^r Cm^r	[36]
pGS058	pACYC184 containing *oxyR2* [A233V]	[36]

Table 3.2 Primers

Plasmid	Primer sequences
pSUF1	Forward 5'-ATATCGAATTCTAAGTAAGAGGTAAATCGATGGACA-3' Reverse 5'-CATGGATCTAGATTAGCTAAGTGCAGCGGC-3'
pISC1 pISC2	Forward 5'-ATATCGAATTCTTTAATACGGAGTTTATAGAGCA-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pIscS-$(His)_6$	Forward 5'-ATCGATCCATATGAAATTACCGATTTATCTCGAC-3' Reverse 5'-CATATAGGATCCTTAATGATGAGCCCATTCGATG-3'
pIscSUA	Forward 5'-ATATCGAATTCTTTAATACGGAGTTTATAGAGCA-3' Reverse 5'-CATGGATCTAGATCAAACGTGGAAGCTTTC-3'
pSJ104	Forward 5'-ATATCGAATTCTTTAACGCAGCCCTGAGAATGTT-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pSJ107	Forward 5'-ATATCGAATTCTTTAACCGTGGACGAGGTTTAAT-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pPsuf	Forward 5'-ATATGCCTGCAGCTTAAGGGTTTTCTTATTTC-3' Reverse 5'-TATACCGGTACCCATCGATTTACCTCACTTC-3'
pPsufNI	Forward 5'-ATATGCCTGCAGCTAACAATGAGATACCTAATTC-3' Reverse 5'-TATACCGGTACCCATCGATTTACCTCACTTC-3'
pPiscR	Forward 5'-ATATGCCTGCAGAGGTCGGATAAGGCGTTC-3' Reverse 5'-TATACCGGTACCCATGTCTTACTTCACCTC-3'
pPiscRS	Forward 5'-ATATGCCTGCAGAGGTCGGATAAGGCGTTC-3' Reverse 5'-TATACCGGATCCCATTGCTCTATAAACTCC-3'
pPleuL2	Forward 5'-ATATGCCTGCAGTACTTAACTCCACTGTCA-3' Reverse 5'-TATACCGGTACCCTGTTCACCGTCGCGCAATG-3'T
pFdx-$(His)_6$	Forward 5'-ATCGATCCATATGCCAAAGATTGTTATTTTGCCTC-3' Reverse 5'-CATATAGGATCCTTAATGCTCACGCGCATGGTTG-3'

3.6 FIGURES

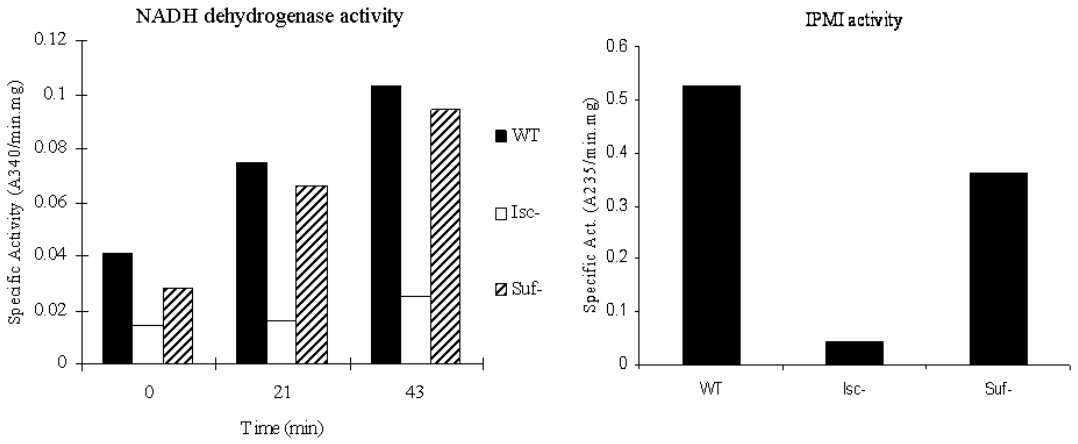

Figure 3.1 The Isc system is the major [Fe-S] cluster under a normal condition. To measure the activity of NDAH dehydrogenase, WT, hscA, and suf cells were grown anaerobically prior to an aerobic growth in glucose/CAA minimal media. The activities were measured at the designated time points. The samples at time zero were prepared from anaerobic cultures. IPMI activities were measured from aerobic cultures that were grown in glucose/18 a. a. (-Leu and -Cys) minimal A media.

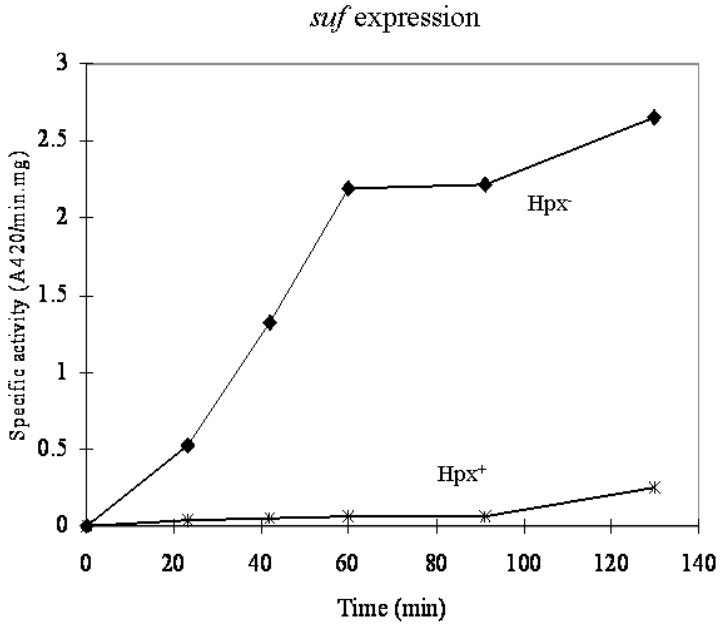

Figure 3.2 The *suf* operon is induced by sub-micromolar H_2O_2 (0.5 – 1 μM). Cell were grown anaerobically prior to an aerobic growth. The induction of the *suf* operon was monitored by transcriptional *lacZ* fusions that behind the *suf* promoter. Anaerobic cultures were also harvested as zero-time points.

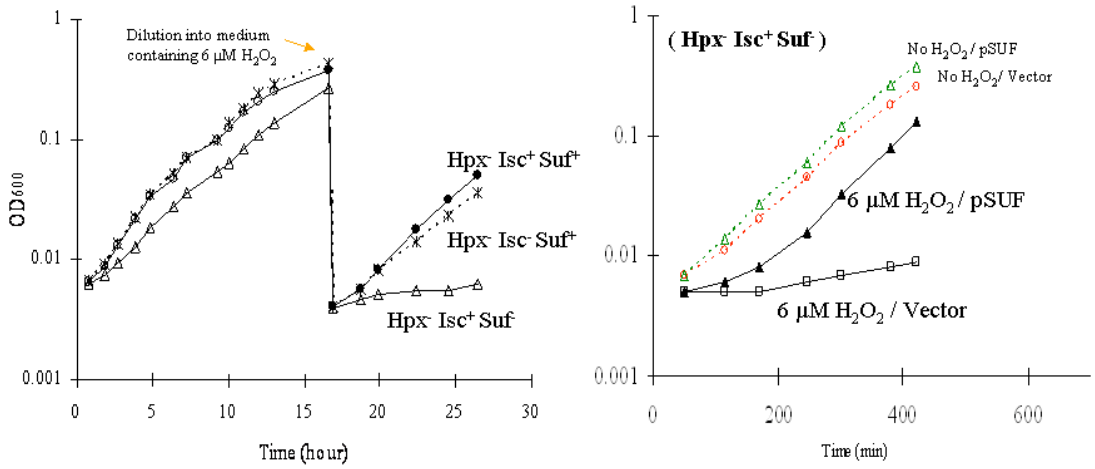

Figure 3.3 The suf mutant showed growth defects during H_2O_2 stress, and the overexpressed Suf system relieved the growth defect. To induce the Suf expression, cells were grown aerobically in glucose/His/aromatic a.a. minimal A medium, and diluted to OD_{600} = 0.005 in flesh media containing 6 μM H_2O_2. The complementation experiment was performed in an aerobic medium with the Suf overexpression plasmid.

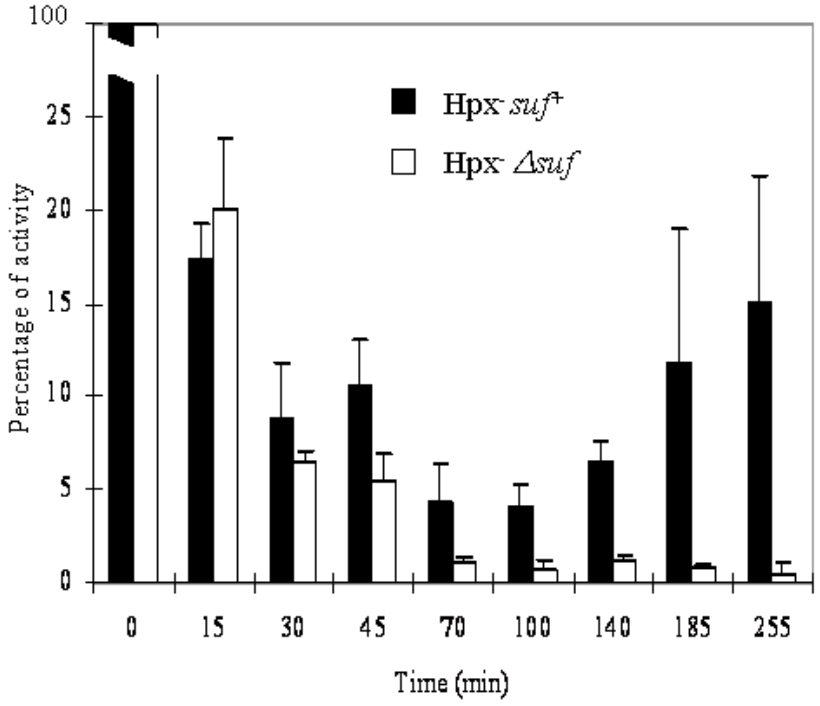

Figure 3.4 Induction of the Suf system increases IPMI activity during H_2O_2 stress. Anaerobically grown cells were aerated at time zero. IPMI activity was monitored during aerobic growth.

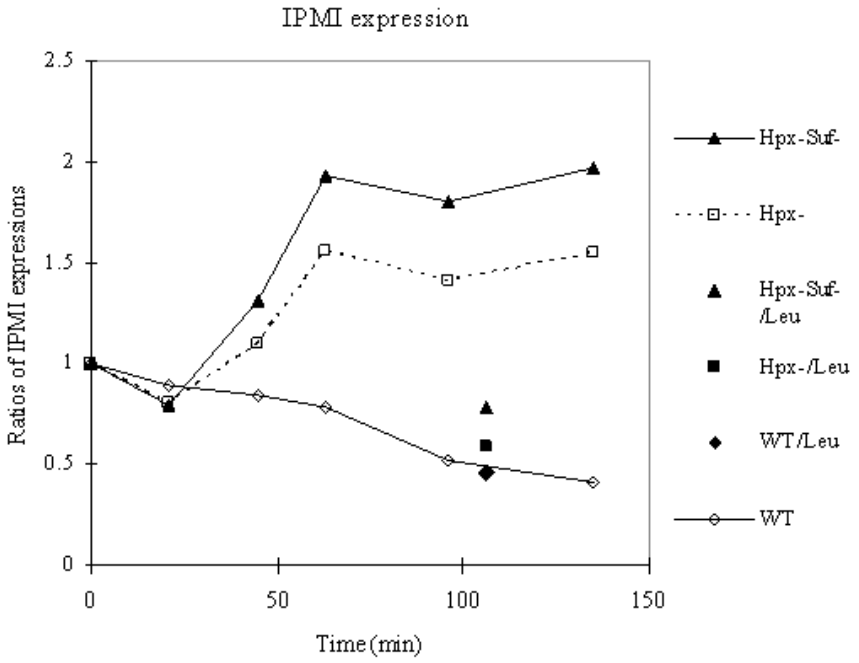

Figure 3.5 The expression of IPMI was increased during H_2O_2 stress. Cell were grown anaerobically prior to an aerobic growth. The induction of the *leuCD* operon was monitored by transcriptional lacZ fusions that behind the *leuL* promoter. Anaerobic cultures were also harvested as zero-time points. The construction of the transcriptional fusion was confirmed by decreased *leuCD* transcription upon the addition of leucine (0.5 mM).

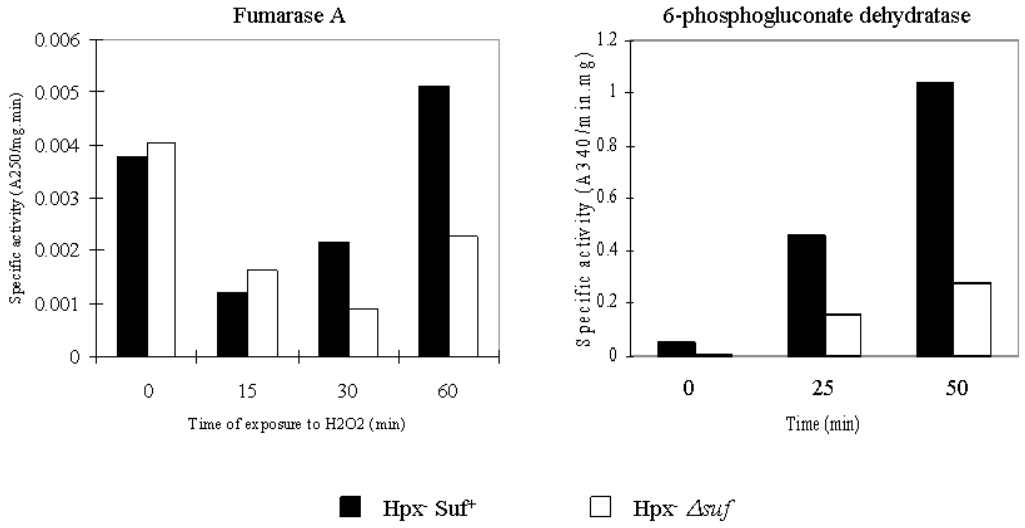

Figure 3.6 The Δsuf mutant also showed low activities of other dehydratases that contain labile [4Fe-4S] clusters. Cells were grown anaerobically prior to aerobic growth. In the case of fumarase A, additional 20 uM H2O2 was added when cells were aerated. To measure 6-phosphogluconate dehydratase, cells were anaerobically grown in a glucose/CAA minimal A medium. Then, they were resuspended in an aerobic gluconate medium.

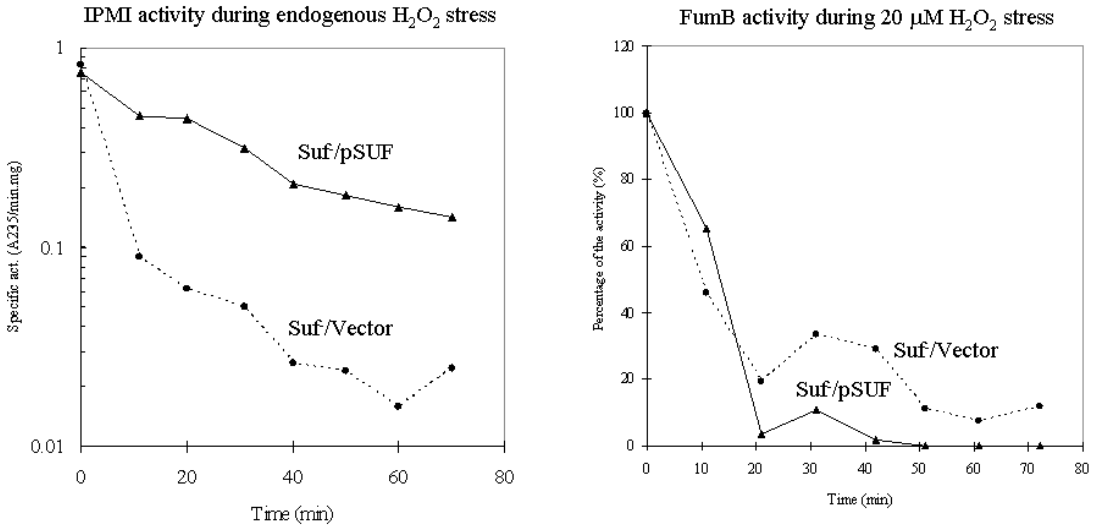

Figure 3.7 The Suf system repairs damaged IPMI but not FumB. Hpx$^-$ *Δsuf* cells were grown anaerobically either with pSUF or an empty vector in a lactose/18 a. a. (-Leu and –Cys) minimal A medium. Chloramphenicol was added to block the protein synthesis prior to aeration of the cultures. In the case of FumB, 20 μM H_2O_2 added when the culture was aerated. The activities were monitored at the time points designated.

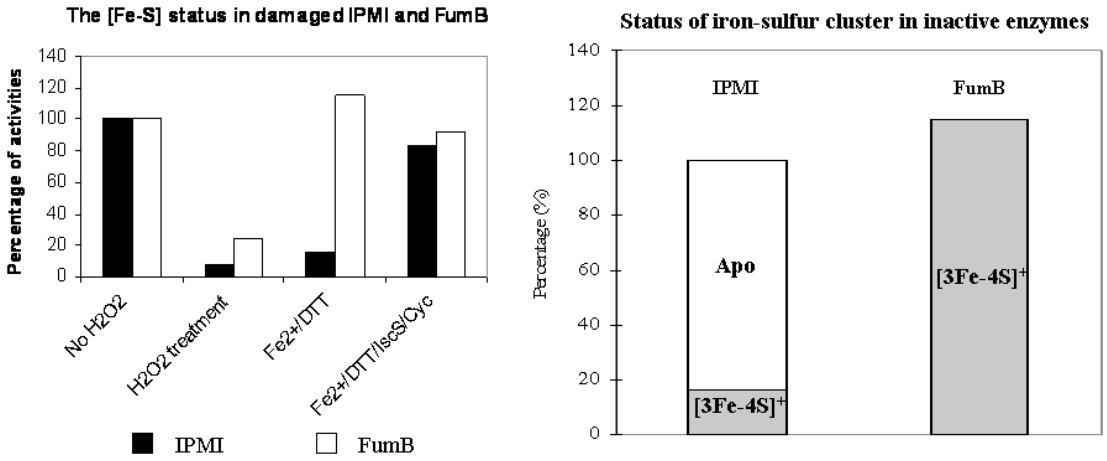

Figure 3.8 The cluster in damaged IPMI is beyond a [3Fe-4S] status unlike fumarase. The status of damaged cluster in IPMI and fumarase was determined by either chemical repair or reconstitution of [Fe-S] cluster. Lysates from Hpx$^-$ *Δsuf* cells that had damaged IPMI or FumB were incubated with either Fe^{2+}/DTT or Fe^{2+}/DTT/IscS/Cys at room temp. The activity was normalized to the activity from cell lysates, of which cells were not treated with H_2O_2.

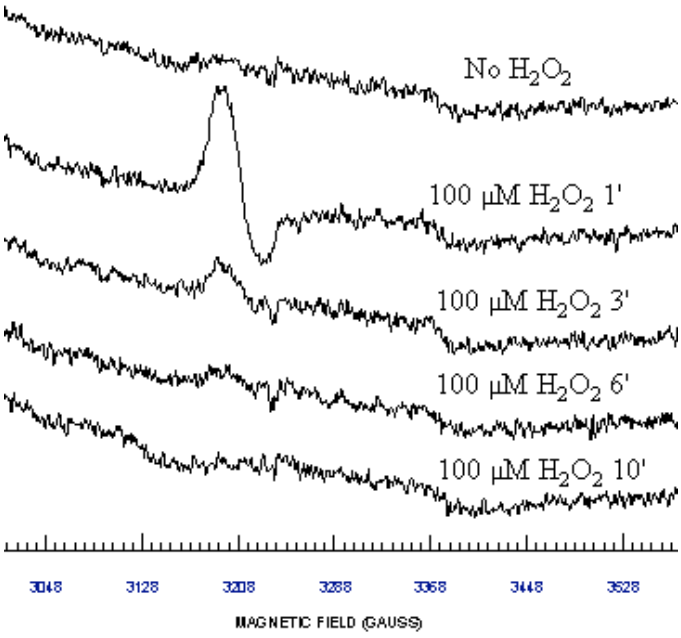

Figure 3.9 The [3Fe-4S]$^+$ cluster in damaged IPMI was further degraded beyond a [3Fe-4S] status. Hpx- *Δsuf* cells, in which IPMI was overexpressed from the plasmid, were treated with H_2O_2. Cells were prepared for EPR analysis at the time points designated.

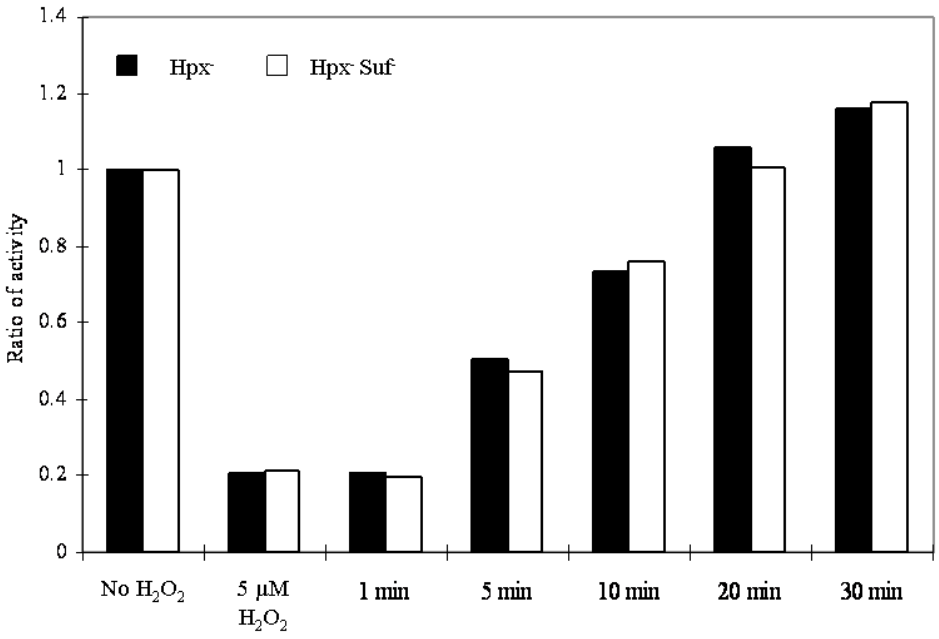

Figure 3.10 The Suf system is not involved in repair of [3Fe-4S]$^{+}$ clusters. Purified FumA was inactivated with 5 µM H_2O_2 for 5 min in ice. Inactivation was ceased by addition of catalase. Crude extracts from either Hpx^{-} or Hpx^{-} *Δsuf* cells were added to inactivated FumA. The activity of FumA was monitored overtime.

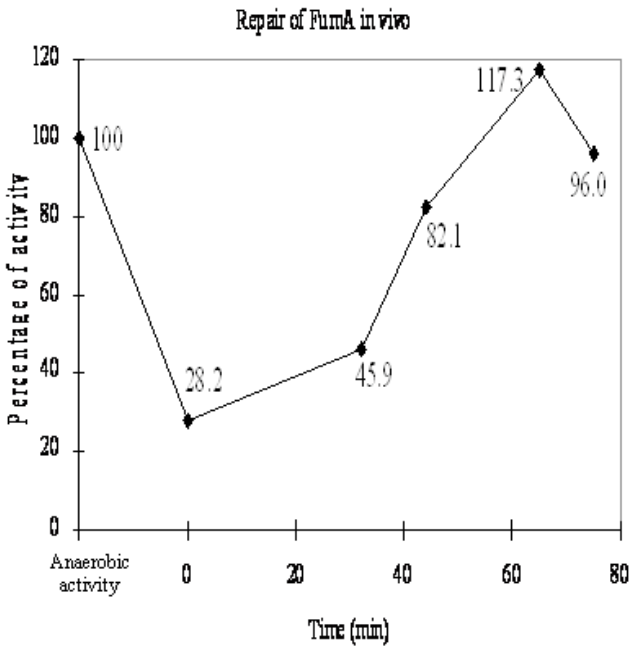

Figure 3.11 In vivo the repair of $[3Fe\text{-}4S]^{+}$ cluster in FumA does not depend on the Suf system. Cells were grown anaerobically in glucose/CAA minimal A medium. Chloramphenicol was added to the culture prior to aeration with 20 μM H_2O_2 for 5 min. Catalase was added subsequently to remove H_2O_2. The FumA activity was monitored over time. The activities were normalized to anaerobic FumA activity. The time zero represents the residual activity after H_2O_2 treatment.

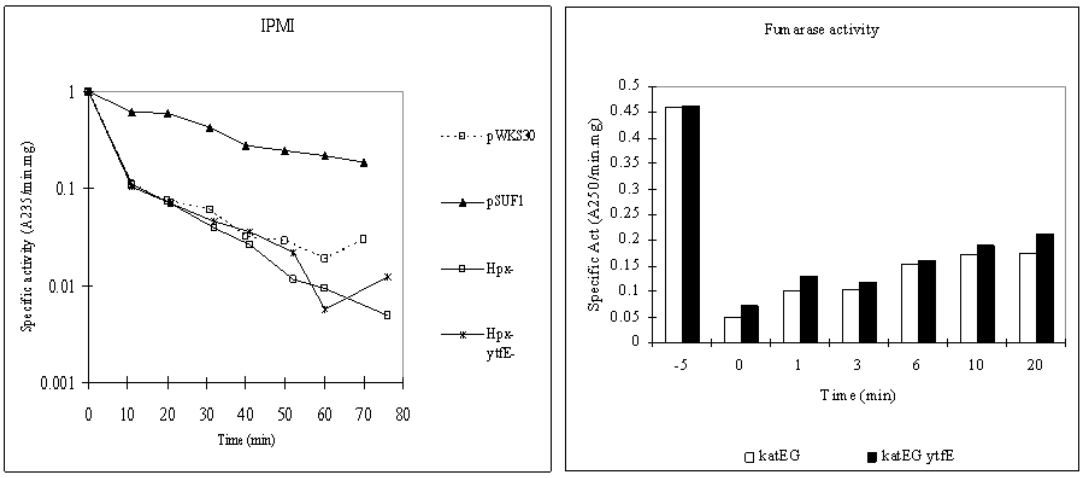

Figure 3.12 The potential di-iron protein, YtfE neither interferes the repair process of the Suf system nor repairs [3Fe-4S]$^{+}$ clusters. Cells were grown anaerobically in a glucose/CAA medium. Chloramphenicol was added to block the protein synthesis prior to aeration of the cultures. IPMI activity was monitored during aeration without exogenous H_2O_2. The repair of damaged FumA was examined in a catalase mutant (*katEG*). Cells were grown aerobically in a glucose/CAA minimal A medium. Chloramphenicol was added prior to addition of 2 mM H_2O_2. The H_2O_2 treatment was ceased after 5 min by adding catalases. The activity of FumA was monitored over time.

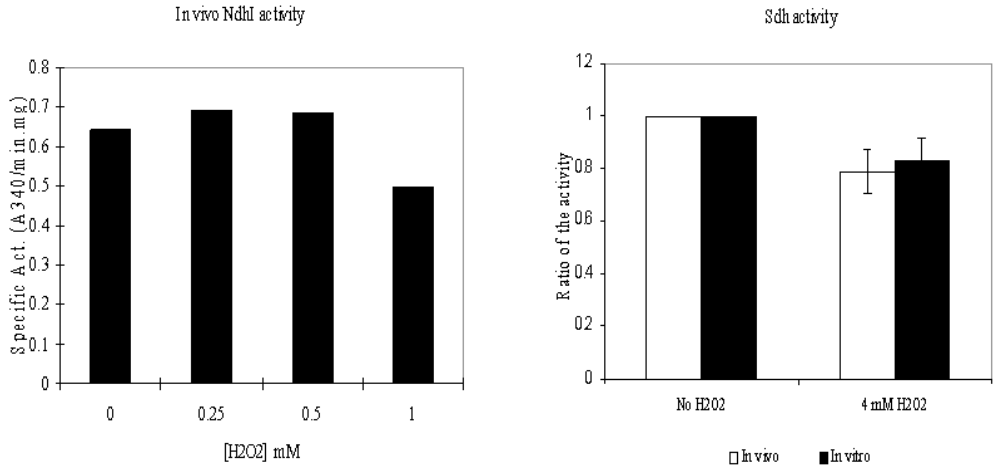

Figure 3.13 The [Fe-S] clusters in NADH dehydrogenase (Ndh1) and succinate dehydrogenase (Sdh) are resistant to H_2O_2. A *katEG* mutant was grown aerobically in a glucose/CAA minimal A medium. Chloramphenicol was added to inhibit the protein synthesis. To examine the H_2O_2-resistance in vivo, various concentrations of H_2O_2 were added to cultures and cells were harvested after 5 min incubation with H_2O_2. In vitro H_2O_2-resistance of the enzymes was also investigated in lysates of the cells that were not treated with H_2O_2.

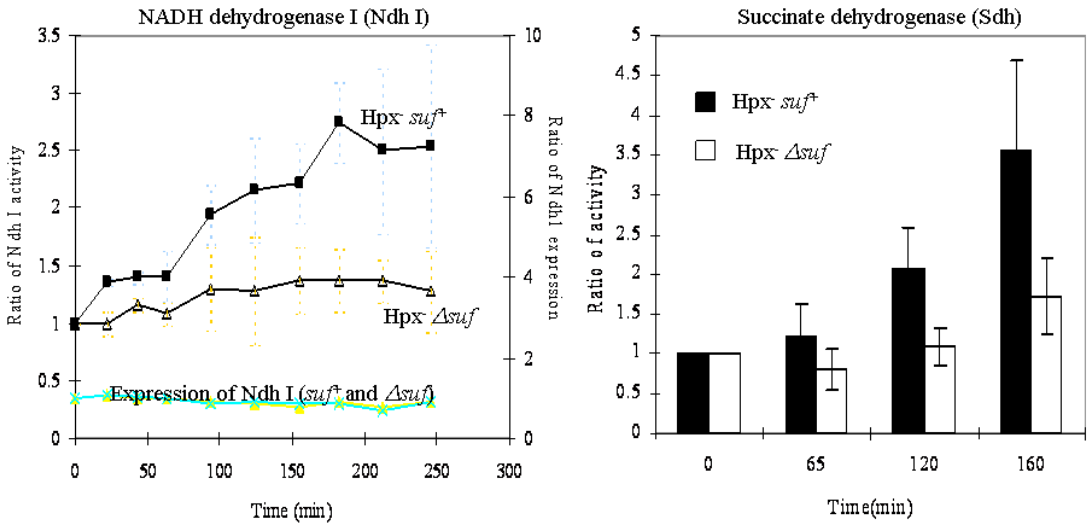

Figure 3.14 The Suf system is the major [Fe-S] cluster assembly system during H_2O_2 stress. The activities of NADH dehydrogenase and succinate dehydrogenase were measured in Hpx$^-$ *suf*$^+$ and Hpx$^-$ *Δsuf* cells. The cells were grown anaerobically in a glucose/CAA minimal A medium prior to aeration. Expression of Ndh1 and Sdh were also monitored by the transcriptional *lacZ* fusions under *nuoAB* and *sdhC* promoters.

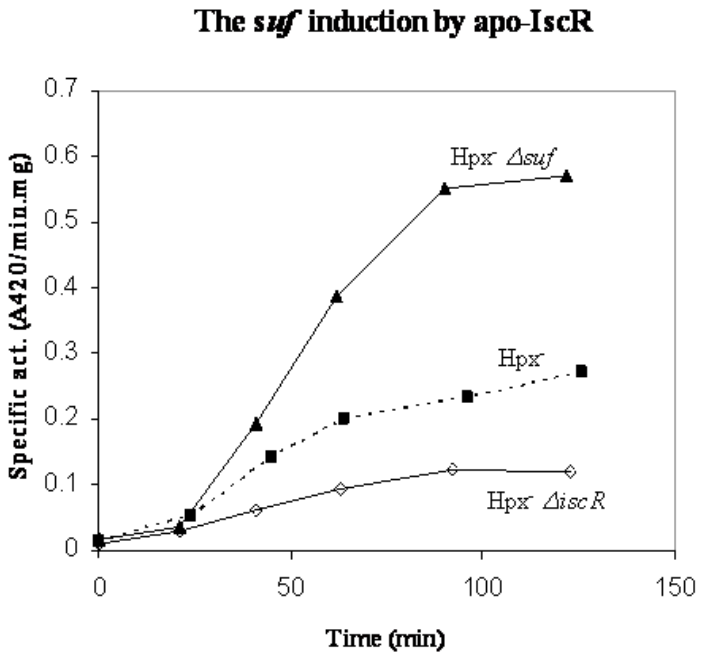

Figure 3.15 During H_2O_2 stress, apo-IscR accumulated due to the inactivation of the Isc system. The accumulation of apo-IscR was monitored by measuring the induction of the *suf* operon by apo-IscR. The transcriptional *lacZ* fusion was constructed behind the *suf* promoter, in which the OxyR binding site was removed. Initially, cells were grown anaeribically in a glucose/CAA minimal A medium. Aerobic growth began with aeration of anaerobic cultures (0 time).

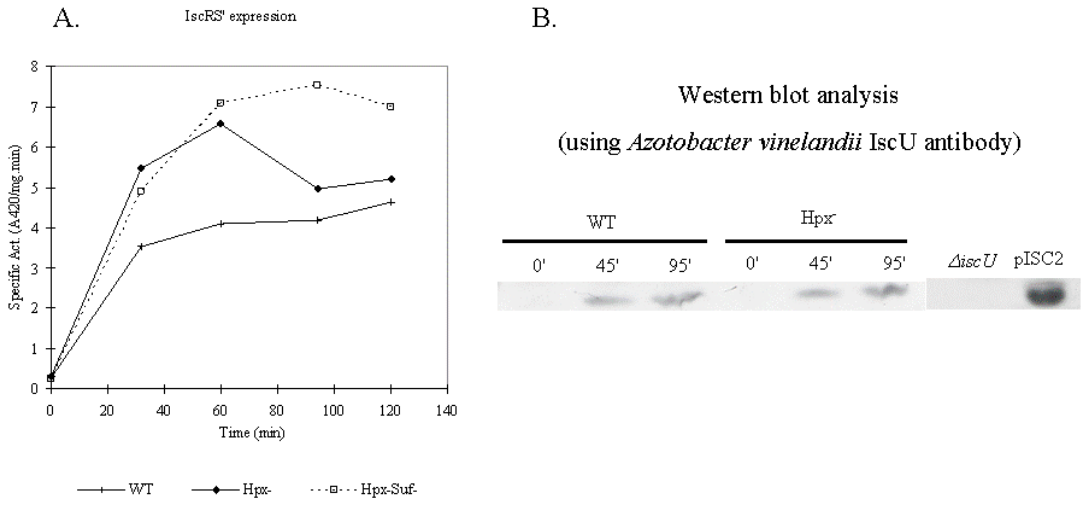

Figure 3.16 The Isc system is expressed during H_2O_2 stress. The transcriptional *lacZ* fusion was constructed behind the *iscRSUA* promoter. The cells that contained the fusion were grown anaerobically prior to aeration. The levels of the transcription were monitored by measuring β–galactosidase activity. The IscU polypeptide was also detected by western blot analysis using anti-IscU antibody.

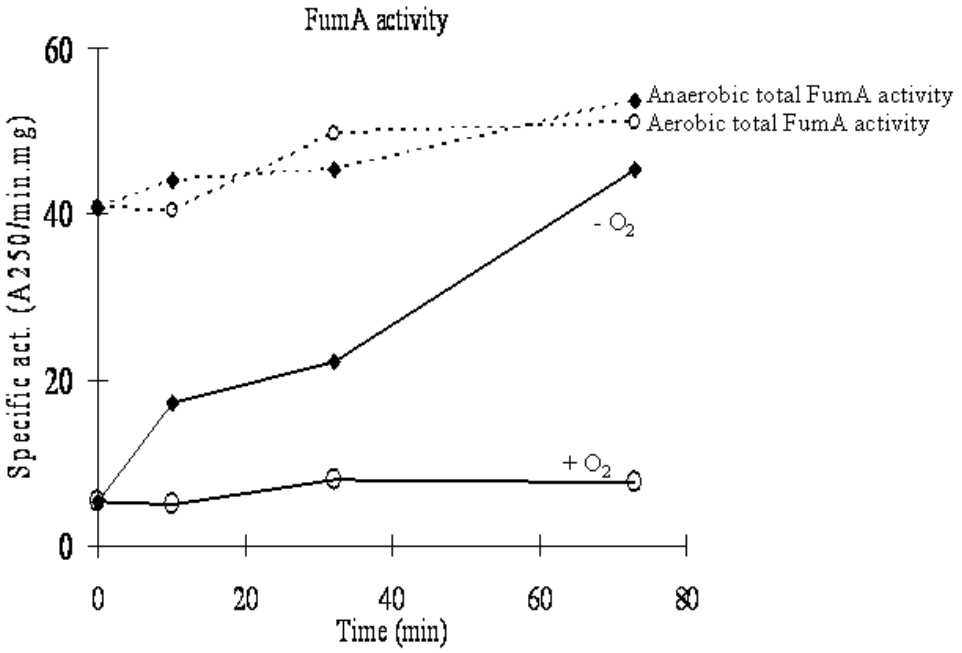

Figure 3.17 Inactivation of the Isc system is reversible. FumA was overexpressed in aerobically grown Hpx$^-$ *Δsuf* cells with 1 mM IPTG. Chloramphenicol was added to inhibit the protein synthesis. Then, the culture was split into two; each split culture was incubated either aerobically or anaerobically. Catalase was added for the anaerobic incubation. At the time points, given cells were harvested and FumA was assayed. Total FumA activity was determined by reconstitution of [Fe-S] clusters using Fe^{2+}/DTT/IscS/Cys.

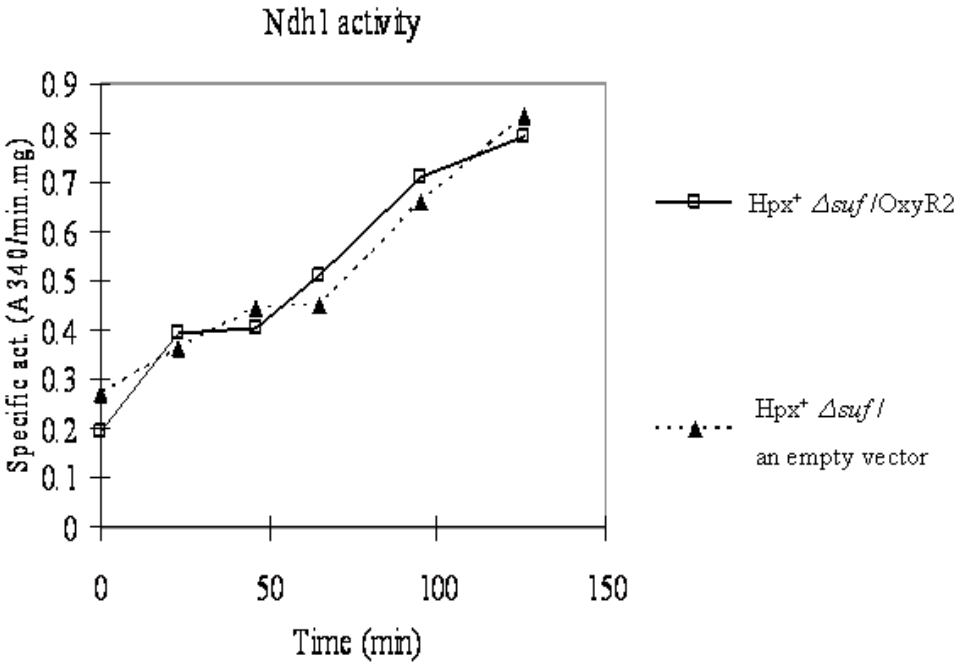

Figure 3.18 Induction of the OxyR regulon did not inactivate the Isc system. Cells were grown anaerobically in glucose/CAA minimal A medium to OD_{600} = 0.1 prior to aeration. Newly synthesized NADH dehydrogenase I was measured during aerobic growths.

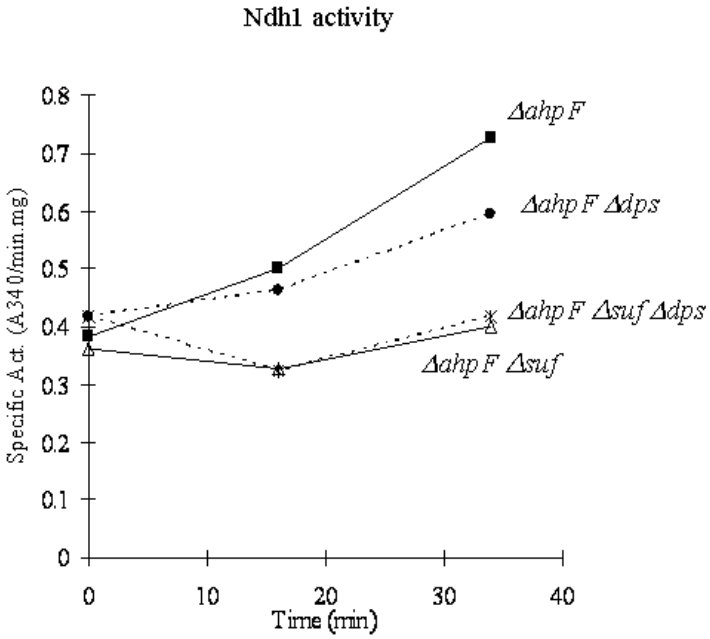

Figure 3.19 Increased Dps expression did not inhibit Isc system function. Exogenous 20 μM H_2O_2 was added to anaerobic cultures before aerobic growths started. NADH dehydrogenase was measured at designated time points.

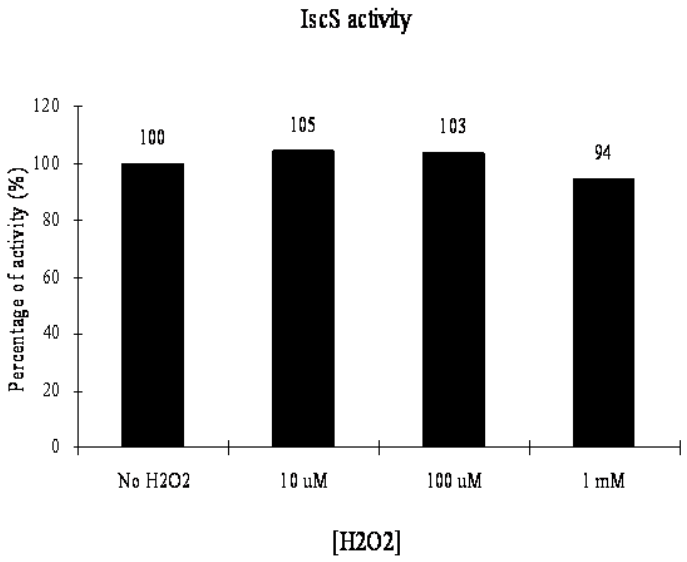

Figure 3.20 H_2O_2 did not inactivate IscS. Purified IscS-$(His)_6$ was treated with various concentrations of H_2O_2 anaerobically. Catalase was added after 5 min challenge to remove H_2O_2. Desulfurase activity of IscS was measured as described in Materials and Methods.

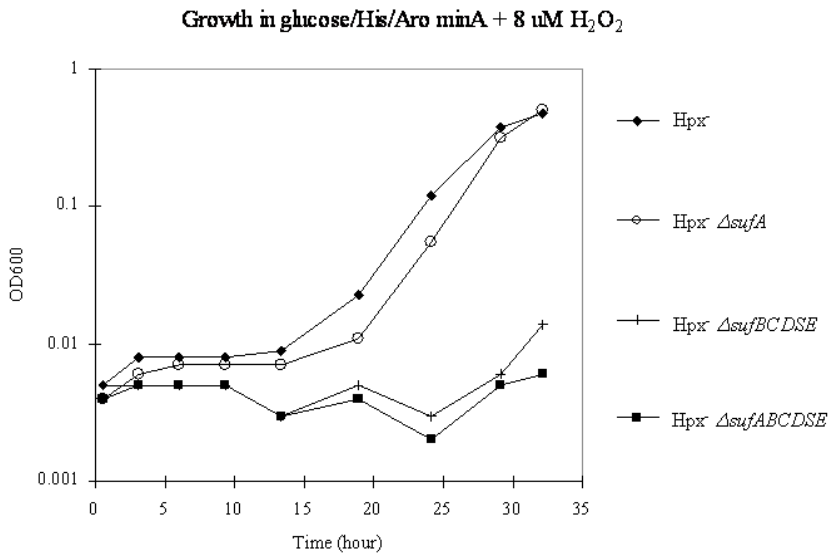

Figure 3.21 SufA can be replaced with IscA , indicating that IscA is functional during H_2O_2 stress. Cells were grown anaerobically in glucose/His/Trp/Tyr/Phe minimal A medium to OD_{600} = 0.1. Then, the cells were diluted to OD_{600} = 0.05 in aerobic fresh medium that contains 8 μM H_2O_2. Growth rates were monitored at OD_{600}.

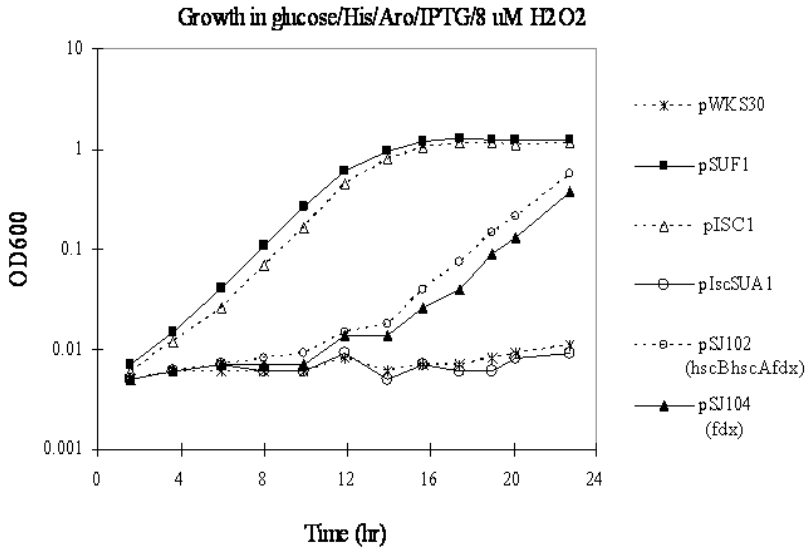

Figure 3.22 The overexpression of individual Isc proteins, except Fdx, did not complement the growth defect of an Hpx$^-$ suf mutant, while the overexpressed Isc complex did. Cells were grown anaerobically in glucose/His/Trp/Tyr/Phe minimal A medium containing 50 µg/ml ampicillin to OD_{600} = 0.1. IPTG (1 mM) was added to anaerobic cells that had plasmids in order to induce Isc proteins prior to aerobic growth. Then, the cells were diluted to OD_{600} = 0.05 in aerobic fresh same-composition medium that contained 8 µM H_2O_2. Growth rates were monitored at OD_{600}.

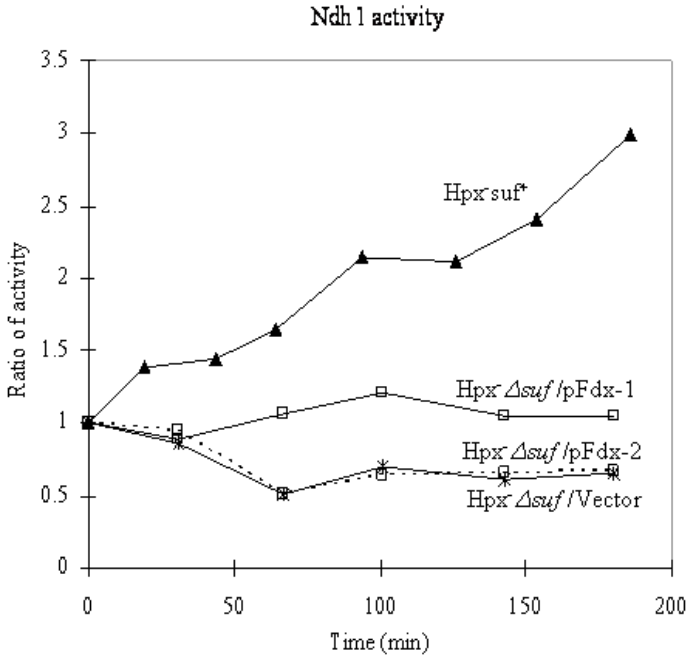

Figure 3.23 The overexpressed Fdx did not restore Isc function. Cells were grown anaerobically in glucose/CAA minimal A medium, and the aeration started at time zero. IPTG (1 mM) was added to anaerobic cells that had plasmids in order to induce Fdx prior to aerobic growths. At designated time points, cells were harvested and NADH dehydrogenase was measured.

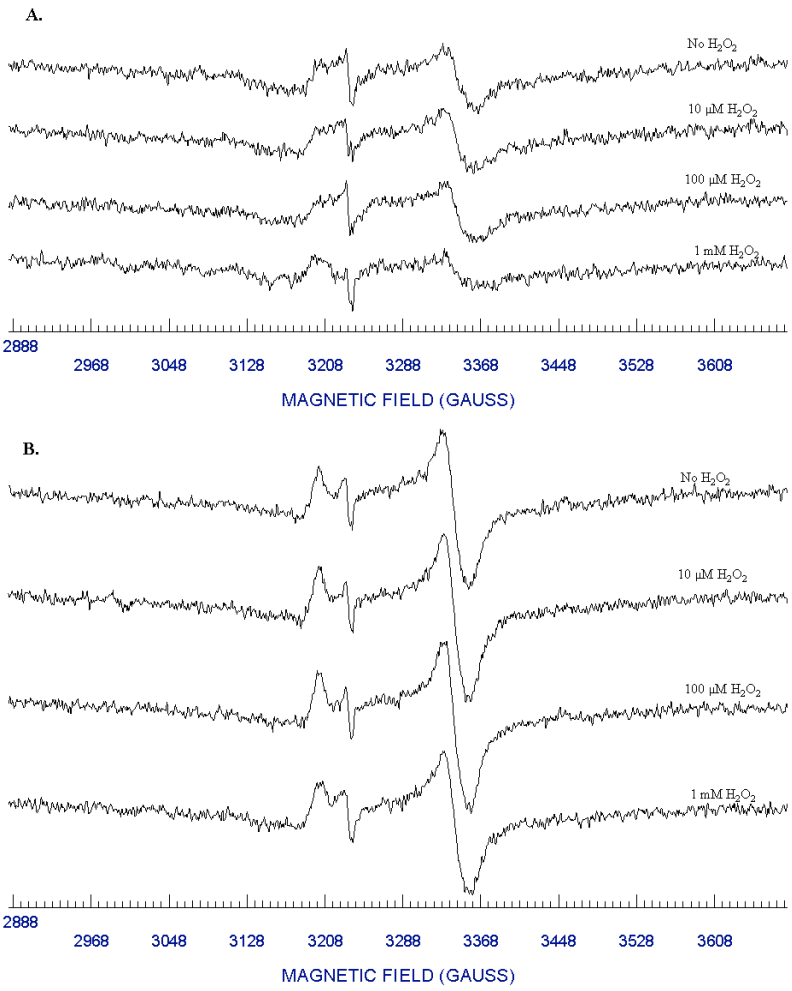

Figure 3.24 Neither oxidation nor decrease of [Fe-S] clusters were detected by EPR analysis in the overexpressed Isc system upon in vivo H_2O_2 treatment. The Isc system was overexpressed under the control of *tac* promoter in Hxp$^-$ cells by 1 mM IPTG. Anaerobically, cells were harvested and treated with various concentrations of H_2O_2 at room temp for 5 min. Catalase was added to remove H_2O_2. Each sample was split into two, one of them were immediately transferred to an EPR tube, and frozen in dry ice for EPR analysis (A). 10 mM dithionite was added to another half of a sample (B). After 5 min incubation at room temp, dithionite treated cells were transferred to EPR tubes and frozen in dry ice. EPR analysis was performed as described in Materials and Methods.

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CHAPTER 4: CONCLUSIONS

4.1 SUMMARY OF CURRENT WORK

4.1.1 Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes

In this study, we wish to identify the biomolecules that are physiologically sensitive to H_2O_2. To do so, an *E. coli* catalase/peroxidase mutant (Hpx^-) was examined as a model system. We found that a leucine auxotrophy appeared when low concentrations of extracellular H_2O_2 were added to Hpx^- cultures. The cause of the leucine auxotrophy was then tracked down. In vivo and in vitro experiments showed that in Hpx^- cells, isopropylmalate isomerase in the leucine biosynthetic pathway is inactivated due to oxidation of its solvent-exposed [4Fe-4S] cluster to an inactive [3Fe-4S] cluster by H_2O_2. Similar H_2O_2 sensitivities were found in [4Fe-4S] clusters of other dehydratases, such as fumarases and 6-phosphogluconate dehydratase, indicating that solvent-exposed [4Fe-4S] clusters are primary targets of H_2O_2. The rate constant with which H_2O_2 damages a [4Fe-4S] cluster in vivo was 4×10^4 at 37 °C, which is comparable to the rate constant with which H_2O_2 oxidizes ferrous iron on DNA.

4.1.2 Hydrogen peroxide poisons the *Escherichia coli* Isc cluster assembly system, and the Suf system is induced to compensate

In vivo [Fe-S] clusters are synthesized enzymatically by assembly systems. *Escherichia coli* has two [Fe-S] cluster assembly systems: the Isc (iron-sulfur cluster) and Suf (sulfur mobilization) systems, whose double mutation is lethal [11]. The fact that the global H_2O_2 sensor, OxyR, regulates the *suf* operon [14] has implied that the Suf system

may play an important role during H_2O_2 stress. We hypothesized that the Suf system is needed because the Isc system does not work during H_2O_2 stress. Indeed, a few micromolar H_2O_2 caused growth defects and low activities of [4Fe-4S] cluster containing dehydratases in a *suf* mutant but not in an *isc* mutant. Furthermore, Hpx^- *Δsuf* mutants failed to synthesize [Fe-S] clusters in NADH dehydrogenase and succinate dehydrogenase even without exogenous H_2O_2. These results showed that very low concentrations of H_2O_2 (0.5-1 μM) impair the function of the Isc system and that the Suf system is necessary to compensate for the loss of the Isc system. The mechanism by which H_2O_2 poisons the Isc system is still unclear.

4.2 POSSIBLE FUTURE WORK

4.2.1 Why are [4Fe-4S] clusters of dehydratases less sensitive to H_2O_2 in vivo than in vitro?

Although 20 μM exogenous H_2O_2 is needed to create in vivo inactivation of fumarase A, purified fumarase A shows great sensitivity to H_2O_2 (4×10^3 $M^{-1}s^{-1}$ at 0ºC) in vitro. Similarly, aconitase A is resistant to oxidants in vivo, but it becomes vulnerable to H_2O_2 once it is purified [13]. There are a few possibilities that could explain these discrepancies between in vivo and in vitro: 1) substrate protection, and 2) in vivo repair.

In vivo and in vitro assays show that [4Fe-4S] clusters in dehydratases can be completely protected by saturating amounts of substrates (Chapter 2). Substrates likely play a role in protection of dehydratases in vivo, since the inactivation of dehydratases would cause their substrates to accumulate. This idea can be tested using a *leuA* mutant

that lacks the enzyme upstream of IPMI. If the substrates protect IPMI in vivo, a *leuA* mutant would show a higher inactivation rate constant compared with *leuA*$^+$ cells.

Alternatively, in vivo repair processes that do not happen in vitro might be responsible for the discrepancies. When damage to [4Fe-4S] clusters occurs, inactive [3Fe-4S]$^+$ clusters are generated. In vivo experiments indicated that cells can repair damaged [3Fe-4S]$^+$ clusters (Chapter 3). The [3Fe-4S]$^+$ clusters can be reconstituted in vitro by addition of ferrous iron and reductants, such as dithiothreitol. Therefore, in vivo repair is likely performed by iron donors and reductants. OxyR induces TrxC and GrxA [14], which are potential electron donors for the repair process. Since iron metabolism is not completely understood, the source of iron for the repair process has not been identified. Although several candidates have been suggested, there is little compelling experimental evidence. YggX, a possible iron-trafficking protein, is induced by SoxRS [10]. It has been reported that an *yggX* mutant showed decreased aconitase B activity. Our lab also has seen that *yggX* mutants showed lower dehydratase activity after paraquat treatments, probably due to impaired repair (M. Gu's unpublished data).

4.2.2 How do the further degradation of a [3Fe-4S] cluster and the cleavage occur on IPMI?

So far, IPMI is the only enzyme we have identified, in which a damaged [3Fe-4S] cluster is degraded beyond a [3Fe-4S] status and the polypeptide is cleaved. However, in vitro neither the further degradation of a [3Fe-4S] cluster nor the polypeptide-cleavage occurred upon H_2O_2 treatments, suggesting that the degradation of a [3Fe-4S] cluster triggers the cleavage of the polypeptide (Chapter 3 and Appendix II). One could speculate that apo-LeuC might be misfolded and proteases degrade it. This

idea can be explored by mutating cysteine residues in LeuC that would create apo-LeuC. We would then monitor whether the mutant protein is cleaved in wild-type cells.

Alternatively, the cleavage may be caused by a hydroxyl radical that is adventitiously produced near the active site. The in vivo turnover number of IPMI damage is more than one due to the repair process, while the in vitro turnover number is only one. Therefore, it is possible that in vivo many cycles of damage temporarily increase the local-iron concentration nearby IPMI, producing hydroxyl radicals by Fenton chemistry. Hydroxyl radicals then cleave the polypeptide chain. If so, other injuries such as carbonylation, which a hydroxyl radical causes, would also likely occur on IPMI. These injuries can be detected by mass spectrometry analysis.

Also, whether other enzymes behave like IPMI would be worthy of investigation.

4.2.3 Is a [2Fe-2S] cluster in dehydratase sensitive to H_2O_2?

A solvent-exposed [4Fe-4S] cluster is, to date, the only class of [Fe-S] cluster that is known to be sensitive to H_2O_2 [6, 8]. Most [Fe-S] clusters are protected by polypeptides, which inhibit direct interaction between H_2O_2 and [Fe-S] clusters. In some cases, such as ferredoxin and SoxR, H_2O_2 and superoxide can change [Fe-S] cluster redox status but they do not damage clusters [7].

There are dehydratases that use a [2Fe-2S] cluster instead of a [4Fe-4S] cluster: 2-methylcitrate dehydratase (AcnC, *prpD* in *E. coli*) and a plant dihydoxyacid dehydratase [1, 5]. An interesting question is whether these enzymes are sensitive to H_2O_2. In these enzymes, a [2Fe-2S] cluster must be exposed to solvent to conduct catalytic reactions, rendering the possibility of oxidation by H_2O_2. The 2-methylcitrate dehydratase also shows aconitase activity [1]. Therefore, PrpD can be investigated in an *acnAB* mutant.

The expression of plant dihyroxyacid dehydratase from a plasmid in *E. coli* would be another approach to explore sensitivity of [2Fe-2S] clusters in dehydratases.

4.2.4 How does H_2O_2 inactivate the Isc system?

Although we could not demonstrate that H_2O_2 damages a nascent [Fe-S] cluster of IscU, our data suggested that it is most likely. However, as our results alluded, it is not an easy task to demonstrate the hypothesis. First, [Fe-S] containing IscU (holo-IscU) cannot be produced by simple overexpression of IscU, probably because the Isc system works as a complex. Since two other Isc proteins (IscA and Fdx) contain [Fe-S] clusters, it is difficult to investigate the [Fe-S] cluster of IscU by EPR in vivo (Chapter 3). Second, it is possible that H_2O_2 damages a nascent [Fe-S] cluster of IscU only when it is transferred to a recipient protein, during which it would be fully exposed to solvent. In this case, even if we somehow overexpress holo-IscU, most [Fe-S] clusters in IscU would show no sensitivity to H_2O_2, leading us to an incorrect conclusion. Unfortunately, we do not have methods to test these ideas.

4.2.5 How is the Suf system resistant to H_2O_2?

Our study clearly demonstrated that the Suf system is resistant to H_2O_2. It has been speculated that SufBCD complex serves as a scaffold [3], in which a nascent [Fe-S] are likely surrounded by polypeptides. In this case, a [Fe-S] cluster in SufBCD would be protected from damage by H_2O_2. Still, it is not clear how a [Fe-S] cluster of Suf BCD could be transferred to client apo-proteins without exposure of the cluster to dissolved oxidants. Other groups are attempting to answer this question biochemically.

4.2.6 How are [Fe-S] clusters transferred from assembly systems to recipient proteins?

Previous in vitro studies showed that [Fe-S] clusters can be transferred to pre-made apo-proteins, such as aconitase, ferredoxin, and biotin synthase [2, 9, 12]. We also showed that [Fe-S] clusters can be reconstituted in vivo and in vitro into apo-fumarases and apo-IPMI. These results suggested that [Fe-S] clusters can be transferred post-translationally. These proteins each have only one [Fe-S] cluster. Therefore, it would be interesting to test whether enzymes that have multiple [Fe-S] clusters can receive clusters post-translationally. This is a more challenging problem for the proteins that contain multiple [Fe-S] clusters, since it seems likely that their apo-forms will be substantially misfolded, requiring chaperons. Another possibility would be the co-translational transfer of [Fe-S] clusters. These ideas can be tested using overexpression of succinate dehydrogenase that has three [Fe-S] clusters [4].

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APPENDIX A: RYHB SMALL RNA MODULATES THE FREE INTRACELLULAR IRON POOL AND IS ESSENTIAL FOR NORMAL GROWTH DURING IRON LIMITATION IN *ESCHERICHIA COLI*

The primitive earth was a reducing environment, in which plenty of iron was available to early organisms. For this reason, many ancient proteins incorporated iron as a catalytic cofactor. These iron-containing enzymes are still retained in contemporary organisms in aerobic habitats, imposing two challenges.

First, molecular oxygen oxidizes soluble ferrous iron and precipitates it as ferric hydroxide, which is less available to cells. To cope with this iron limitation, organisms secrete siderophores, iron chelators, and then import iron-bound siderophores back into the cytosol [3, 4]. The second challenge is that high concentrations of intracellular iron are toxic, causing Fenton-reaction-mediated damage [7, 8]. Therefore, cells carefully modulate intracellular-free-iron concentrations through the action of Fur protein (ferric iron uptake regulator). In an iron-replete medium, Fur is metallated with Fe^{2+} and represses the transcription of iron-uptake systems [1]. On the other hand, an iron deficiency causes demetallation of Fur, resulting in derepression of the Fur regulon [8]. RyhB, a small RNA, is one component of the Fur regulon. During iron starvation, RyhB pairs the mRNAs of iron-storage proteins and iron-containing proteins and recruits the RNA degradosome, inducing degradation of the target mRNAs [5, 6]. Since most RyhB targets are the mRNAs of iron-using proteins, it was hypothesized that RyhB is necessary during iron limitation because it inhibits the synthesis of non-essential-iron-containing proteins, increasing the intracellular iron availability for essential-iron-containing proteins.

To test this hypothesis, Massé's group examined growth phenotypes and cell viabilities in wild-type cells and *ΔryhB* mutants. A *ΔryhB* mutant showed a growth defect in iron-limited medium. Lower cell viability was observed in *ΔryhB* cells compared with wild-type cells when cells were in a deep stationary phase, at which iron is potentially depleted. Dr. Massé asked us to measure intracellular-free-iron concentrations to directly address the function of RyhB. To investigate whether RyhB activation increases the intracellular pool of free iron, I measured the free-iron concentration in cells that overexpressed *ryhB*. As we predicted, the overexpression of RyhB increased intracellular free iron concentration (Fig. A.1). This was also true in *Δfur* backgrounds, in which wild-type RyhB is constitutively active, implying that additional copies of RyhB still can increase the iron pool.

A *Δfur* mutant grew worse in iron-replete medium, and introduction of a *ΔryhB* mutation rescued the grow defect. Since the intracellular free-iron concentration of a *Δfur* mutant is much higher than that of wild-type cells, it was postulated that increased Fenton chemistry creates the growth defect in a *Δfur* mutant, and additional *ΔryhB* mutation may lower the free-iron concentration, restoring the growth of a *Δfur* mutant. However, there was no significant difference of intracellular iron concentrations between *Δfur* and *Δfur ΔryhB* mutants (Fig. A.2), indicating that the growth of *Δfur ΔryhB* is not due to the decreased free-iron concentration. It is still unclear how a *ΔryhB* mutation suppresses the growth defect of *Δfur* mutants.

In summary, an intracellular free iron concentration is modulated by the action of Fur and RyhB. During iron limitation, RyhB maintains free iron concentrations by decreasing iron storage proteins and non-essential iron-containing proteins.

A.1 MATERIALS AND METHODS

Intracellular free-iron measurements

Bacterial cultures were grown overnight in LB medium at 37°C and then diluted 1000-fold into 500 ml of fresh LB medium. They were grown at 37°C with agitation to an OD_{600} of 0.5. For cells carrying pNM12 or pBAD-*ryhB* plasmids, arabinose was added to the culture to a final concentration of 0.1% for 15 min. Cells were harvested by centrifugation at 7000 g for 5 min at 4ºC. A cell pellet was resuspended in 8 ml prewarmed fresh minimal medium that contain 10 mM DETAPAC (diethylentriaminepentaacetic acid, pH 7.0) and 20 mM desferrioxamine (pH 8.0). DETAPAC blocks further iron import, while desferrioxamine diffuses into cells and binds unincorporated iron in an EPR-visible ferric form. The concentrated cells were shaken at 37 ºC for 15 min in a water bath. The cells were washed with 5 ml of ice cold 20 mM Tris-Cl (pH 7.4) twice. Cells were then resuspended in 200 µl of ice cold 10% glycerol/20mM Tris-Cl (pH 7.4). The cell suspension (200 µl) then was transferred into an EPR tube and frozen in dry ice. Ferric sulfate standards were mixed with desferrioxamine and prepared in the same Tris buffer containing glycerol. The spectrometer settings were as follows: microwave power, 10 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; temperature, 15 K.

A.2 FIGURES

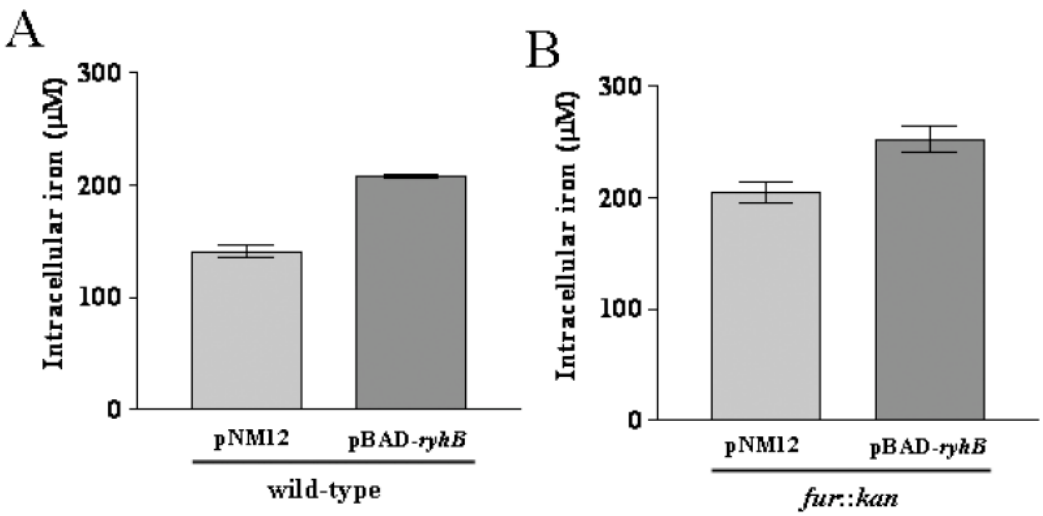

Figure A.1 RyhB expression increases the level of free intracellular iron in wild type (A) and *fur::kan* (B) backgrounds. After diluting the overnight cultures in fresh LB medium, cells (EM1453, EM1456, EM1494 and EM1495) were grown to an OD of 0.5. Arabinose was added at 0.1% to express the sRNA RyhB (pBAD-*ryhB*) or not (pNM12). After 15 min of induction, cells were harvested and treated to measure intracellular free iron.

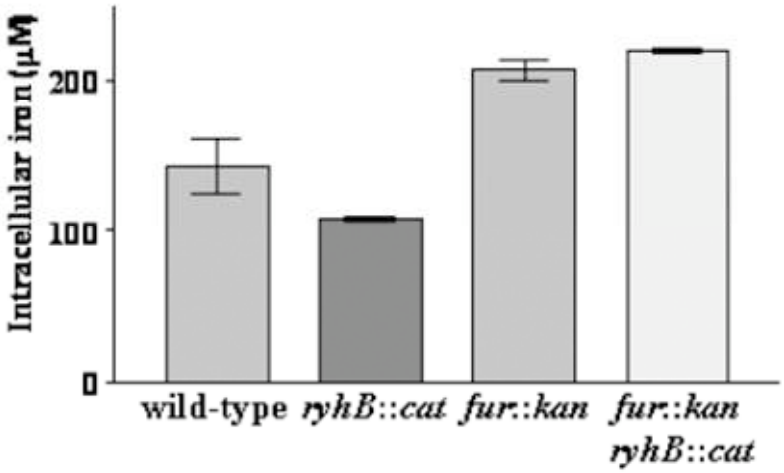

Figure A.2 The growth of *Δfur ΔryhB* mutants is not due to decreased intracellular free-iron concentration. The same growth conditions as in A were used for determining the free intracellular iron of wild type (EM1055), *ryhB::cat* (EM1238), *fur::kan* (EM1256) and *fur::kan ryhB::cat* (EM1257).

APPENDIX B: CLEAVAGE OF IPMI DURING H_2O_2 STRESS

Isopropylmalate isomerase (IPMI) is a dehydratase that is comprised of two proteins, LeuC and LeuD. The catalytic protein, LeuC contains a solvent-exposed [4Fe-4S] cluster in the active site [2]. LeuD has been speculated to be involved in determining substrate specificity [9]. H_2O_2 inactivates IPMI by oxidizing its [4Fe-4S] cluster to an inactive [3Fe-4S] cluster. In Hpx^- cells, over 60% of IPMI is inactivated in the first 20 min of aeration.

In the course of the investigation, we noticed that some portions of damaged IPMI are cleaved during H_2O_2 stress. Western blot analyses were performed using overexpressed LeuCD that has an N-terminal Flag-tag insertion. We found that the cleavage occurs in Hpx^- cells but not in wild-type cells. The estimated size of the cleaved fragment is 49.6 kDa, while the intact LeuC is 54.4 kDa (Fig. B.1).

Interestingly, the cleavage did not occur during H_2O_2 exposure in vitro (Fig. B.2). Concomitantly, a damaged [3Fe-4S] cluster of IPMI was not degraded further in vitro. These results indicated that H_2O_2 does not directly cause the cleavage. The cleavage likely occurs when IPMI is an apo-form, and it may be caused by proteases. The mechanism of the cleavage on IPMI during H_2O_2 stress has not been solved.

B.1 MATERIALS AND METHODS

Plasmid constructions

Open reading frames of *leuCD* were PCR-amplified from *E. coli* MG1655 by using the forward primer 5'-ATATCGAATTCAGAAGGGGTGTAATCATGGATTAT

AAAGATGATGATGATAAAGCTAAGACGTTATAC-3' and the reverse primer 5'- CTGGATCTAGATTAATTCATAAACGCAGGTTG -3'. The PCR products were digested with XbaI and EcoRI and inserted into pWKS30. The plasmid (pLEUCD-flag2) construction was confirmed by restriction/sequencing analyses. LeuCD-flag was expressed in lactose media.

Western blot analysis

Cells that contains pLEUCD-flag2 were grown anaerobically in a lactose/CAA minimal A medium. To inhibit further IPMI synthesis, lactose was removed from the cultures by washing cells with minimal A salt. Cells were then resuspended in an aerobic glucose/CAA medium. During an aerobic growth, cells were harvested and prepared for western blot analysis and IPMI assays. The Flag-tag-fused IPMI was detected using ProteoQwestTM FLAG® Colorimetric Western Blotting Kit.

B.2 FIGURES

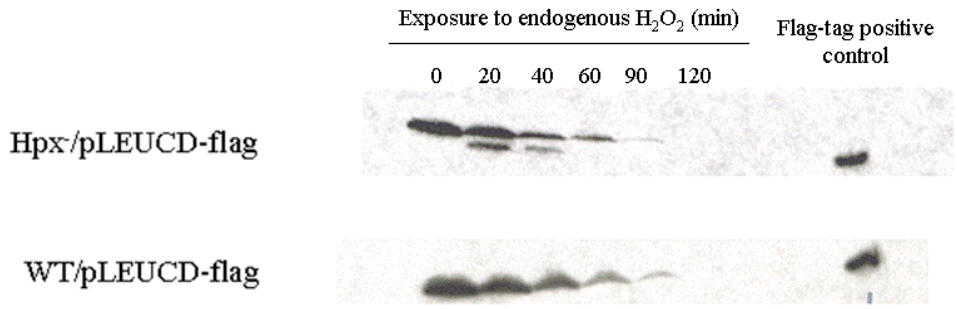

Figure B.1 Some fractions of damaged IPMI were cleaved upon H_2O_2 treatment. The IPMI with flag-tag was expressed under the control of the *lac* promoter in Hpx$^-$ and wild-type cells anaerobically in a lactose/CAA minimal A medium. IPMI synthesis was then inhibited by removing lactose from the medium; cells were washed with minimal A salt prior to dilution into an aerobic glucose/CAA medium. At designated time points, cells were harvested and prepared for western blot analysis.

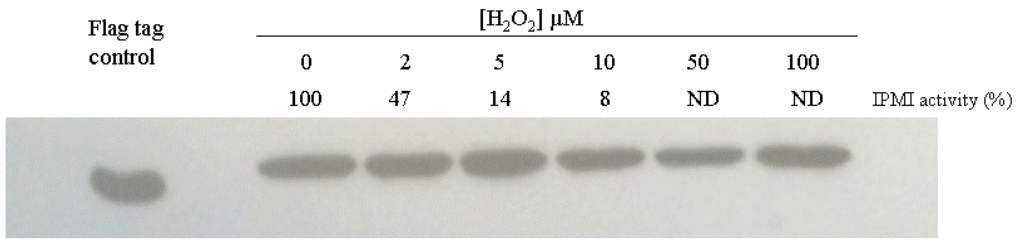

Figure B.2 In vitro H_2O_2 treatment did not cause the cleavage of IPMI. The IPMI with flag-tag was expressed under the control of the *lac* promoter in Hpx$^-$ cells anaerobically in a lactose/CAA minimal A medium. Cells were harvested and a lysate was prepared anaerobically. Various concentrations of H_2O_2 were added to the lysate, and catalases was added after 2 min incubation with H_2O_2. The H_2O_2 treated lysates were prepared for SDS-PAGE analysis, and IPMI activities were measured.

* ND: non-detectible

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CURRICULUM VITAE

Soojin Jang

Education:

University of Illinois Urbana-Champaign, Ph.D. Expected January 2010, Microbiology.
Advisor: James A. Imlay

University of Illinois Urbana-Champaign, M.S. 2005, Microbiology.
Advisor: James A. Imlay

Kyung Hee University, Suwon, Korea, M.S. 2001, Genetic Engineering.
Advisor: Dae Kyun Jung

Kyung Hee University, Suwon, Korea, B.S. 1999, Genetic Engineering.
Advisor: In Sik Jung

Work Experience:

Researcher, RNA Inc., Suwon, Korea May, 2001- June, 2003

Teaching Assistantships:

University of Illinois at Urbana-Champaign:
MCB251, Experimental Techniques in Molecular Biology, Spring 2007
(Rated as an outstanding TA)
MCB251, Experimental Techniques in Molecular Biology, Fall 2006
MCB501, Advanced Biochemistry, Fall 2005

Kyung Hee University, Suwon, Korea:
Microbiology Laboratory, Spring and Fall 2002

Current Research Interests:

Microbial physiology and oxidative stress

Technical Skills:
Molecular Biology:
Development of DNA vectors, cloning, western blots, and southern blots.

Genetics:
Construction of *E. coli* and *Salmonella* mutants, and transcriptional *lac*Z fusions.

Enzymology:
His-tag protein purification, traditional protein purification, determination of rate constants, and many enzyme assays.

Electron paramagnetic resonance spectroscopy of free iron and iron-sulfur clusters.

Publications:
Jang S, Imlay JA. The role of the Suf system during H_2O_2 stress (in preparation).

Jang S, Imlay JA. 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J Biol Chem. 282(2):929-37.

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Cho WJ, Yoon WJ, Moon CH, Cha SJ, Song H, Cho HR, Jang SJ, Chung DK, Jeong CS, Park JW. 2002. Molecular cloning of a novel chaperone-like protein induced by rhabdovirus infection with sequence similarity to the bacterial extracellular solute-binding protein family 5. J Biol Chem. 277(44):41489-96.

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Patent:

Vector for lactic acid bacteria and method for expressing ferritin in the lactic bacteria WIPO Patent Application WO/2002/097098A1

Oral Presentation:

Allerton Research Conference, Department of Microbiology, University of Illinois at Urbana-Champaign, October 2005.

Poster Presentations:

- International Conference on Iron-Sulfur Cluster Biogenesis and Regulation – 5th Annual Meeting (GA 2009).
- Cell and Molecular Biology & Molecular Biophysics Training Grants - 21st Annual Research Symposium (IL 2008).
- Society for Free Radical Biology & Medicine-15th Annual Meeting (IN 2008).
- Gordon Research Conferences: Microbial Stress Response (MA 2006).